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THE IMPACT OF VITAMIN D DEFICIENCY AND SEX HORMONE IMBALANCE ON THE CEREBROVASCULAR SYSTEM

PhD thesis

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List of Abbreviations

AACA: azygous anterior cerebral artery

ACA: anterior cerebral artery

AOC: area over the curve

AR: androgen receptor

CAO: carotid artery occlusion

CoBF: cerebrocortical blood flow

COX: cyclooxygenase

DHT: dihydrotestosterone

eNOS: endothelial nitric oxide synthase

ER: estrogen receptor

MABP: mean arterial blood pressure

MCA: middle cerebral artery

MMP: matrix metalloproteinase

NO: nitric oxide

OVX: ovariectomy

PCOS: polycystic ovary syndrome

PTH: parathyroid hormone

ROS: reactive oxygen species

RXR: retinoid X receptor

TT: testosterone treatment

TXA_2: thromboxane A_2

VDD: vitamin D deficiency

VDR: vitamin D receptor

VEGF: vascular endothelial growth factor

VSMC: vascular smooth muscle cell

WT: wild-type

1. Introduction

1.1. Vitamin D

Vitamin D is well-known for its role in calcium and bone homeostasis, as well as for the adverse consequences of its deficiency on the skeletal system (e.g. poorly mineralized skeleton, growth retardation, increased risk of fractures, decreased muscle strength) (1). However, in the last decades, a great deal of attention has been given to its extraskeletal effects, including the impact on the cardio- and cerebrovascular systems (2).

1.1.1. Biosynthesis and metabolism

Vitamin D is a fat-soluble compound that acts as a steroid hormone (3). Two major forms of vitamin D are distinguished based on the different side chains in their structure: cholecalciferol, also known as vitamin D_3, and ergocalciferol, known as vitamin D_2 (1). Vitamin D_3 is produced in the skin during sun exposure when due to the ultraviolet B (290-315 nm) radiation of the sunlight, 7-dehydrocholesterol non-enzymatically transforms into precholecalciferol (4). Then, as precholecalciferol is unstable, it rapidly isomerizes into cholecalciferol (1). Vitamin D_2 originates from ergosterol produced in plants and fungi (1, 5). Both vitamin D forms go through the same metabolic route in the body to become biologically active, specifically, two hydroxylations (6). Vitamin D is transported in the circulation by vitamin D binding protein to the liver, where it is converted into 25-hydroxyvitamin D by cytochrome P450 enzymes (7, 8). In this step, CYP2R1 is the most important enzyme in humans, while CYP27A1 is also significant in other species (e.g. in mice) (9). The next hydroxylation takes place in the kidneys, where the active 1,25-dihydroxyvitamin D (also known as calcitriol) is formed by the CYP27B1 enzyme (or 1-α-hydroxylase) (3). This step is tightly controlled by 1,25-dihydroxyvitamin D and fibroblast growth factor 23 levels, which can decrease the production of CYP27B1 through transcriptional feedback mechanisms (10). Parathyroid hormone (PTH) is also involved in the mechanism of increasing the renal production of 1,25-dihydroxyvitamin D in response to low serum calcium levels (11). Furthermore, the second hydroxylation can occur in extrarenal tissues, which express CYP27B1 (e.g. in the skin, breasts, placenta, immune cells, and cardiovascular tissues), where 1,25-dihydroxyvitamin D can act as an autocrine/paracrine factor (5, 12). Renal cells also express 24-hydroxylase enzyme (CYP24A1), which is responsible for the conversion of

25-hydroxyvitamin D or 1,25-dihydroxyvitamin D to an inactive, excretable, water-soluble form, and thus balance the level of 1,25-dihydroxyvitamin D (2, 13).

1.1.2. Mechanism of action

The genomic effect of vitamin D is mediated by the vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily (12). When 1,25-dihydroxyvitamin D binds to VDR, they translocate into the nucleus and conformational changes promote the heterodimerization with retinoid X receptor (RXR) (14, 15). The ligand-bound VDR-RXR complex can interact with vitamin D response elements in the promoter region of target genes and thus influence gene transcription (16). VDRs are expressed in numerous organs (e.g. bone, gut, brain) and cell types (e.g. in endothelial cells, vascular smooth muscle cells (VSMC), astrocytes, neurons, cardiomyocytes, and leukocytes), elucidating the ubiquitous role of vitamin D signaling in the body (2, 17, 18).

In contrast to the genomic effects of vitamin D, which take hours or days to develop, its rapid, non-genomic effects mediated by cell surface receptors are exerted within seconds or minutes (18). Non-genomic actions involve the modulation of signaling molecules (e.g. phospholipase C and A_2), protein kinases (e.g. phosphatidylinositol-3-kinase), secondary messengers (e.g. cAMP, calcium), and ion channel activation (e.g. Ca^{2+}, Cl^-) (3, 19). Interestingly, the rapid and nuclear VDR-mediated actions of vitamin D seem to require different ligand conformations (18, 20). Furthermore, it has been reported that the rapid actions of vitamin D may require the presence of nuclear VDR, but controversial evidence also exists (21-24). Although the genomic effects of vitamin D are better elucidated, a number of non-genomic effects have also been described. For example, rapid intestinal calcium absorption, insulin secretion, ion channel responses of osteoblasts, and rapid endothelial cell migration appear to be mediated by non-genomic actions of vitamin D (18). However, the role of rapid actions of vitamin D in the brain, particularly in the vascular system, is less investigated (25).

1.1.3. Sources and optimal status of vitamin D

80-90% of the source of vitamin D comes from sunlight (8, 26). Environmental factors, such as latitude, angle of solar radiation, seasons, and individual factors, like age, skin pigmentation, sunscreen usage, and obesity, can influence vitamin D production (5, 26). Long exposure to sunlight does not cause vitamin D intoxication, as not only the 24-hydroxylase enzyme activity increases with oversupply (4, 8), but the amount of melanin

in the skin also decreases the rate of vitamin D synthesis (6, 16). Foods are another, although less significant, source of vitamin D (1). Cod liver oil, oily fish (e.g. salmon, mackerel, herring), eggs, and mushrooms are high in vitamin D (27). In a few countries, certain products (e.g. milk, margarine, yogurt, cereal) are enriched with vitamin D to decrease the prevalence of vitamin D deficiency (VDD) (4). Besides, dietary supplements or medications are potential options for increasing vitamin D levels (1). Vitamin D can be stored in body fat as being fat soluble, and it can be released later (1).

In clinical practice, serum 25-hydroxyvitamin D concentration is measured to assess vitamin D status (5). The pharmacokinetic properties of this inactive form are more favorable than those of the active dihydroxylated form (2). The half-life of 25-hydroxyvitamin D is around 2-3 weeks in the circulatory system, whereas it is only 4-6 hours for 1,25-dihydroxyvitamin D (6). Serum levels of 25-hydroxyvitamin D are also higher compared to the active form (nanogram *vs.* picogram range), and its quantity is not directly influenced by the above-mentioned factors (e.g. calcium, phosphorus, PTH levels), unlike in the case of 1,25-dihydroxyvitamin D (8). Therefore, in clinical practice, 1,25-dihydroxyvitamin D level is only determined in case of disorders related to 25-hydroxyvitamin D metabolism, for instance, chronic kidney disease, oncogenic osteomalacia, vitamin D-resistant rickets or vitamin D-dependent rickets (28). High performance liquid chromatography, liquid chromatography-tandem mass spectrometry, competitive protein binding, or immunoassays are used to evaluate vitamin D status (8).

Regarding the optimal serum level of 25-hydroxyvitamin D, greater than 30 ng/mL is regarded as sufficient to avoid secondary hyperparathyroidism and bone fracture, and to maximize intestinal calcium absorption (14, 29). Secondary hyperparathyroidism is caused by PTH overproduction due to low 1,25-dihydroxyvitamin D and calcium levels. PTH leads to the stimulation of 1,25-dihydroxyvitamin D formation in the kidneys, and its overproduction results in excessive calcium mobilizing from bones and phosphorus loss in urine (28, 30). Lower levels than 20 ng/mL are considered deficiency, whereas 21-29 ng/mL are considered insufficiency (28, 29). However, it is important to note that the current recommendations aim to maintain proper musculoskeletal health but do not consider extraskeletal health prevention, including obtaining possible cardiovascular benefits (5, 26). Vitamin D intoxication can occur at plasma concentrations above 150 ng/mL. Although it is extremely rare, as the sunlight destroys excessive vitamin D

produced in the skin, intoxication can occur due to inappropriate dietary supplementation (4, 6). Symptoms of intoxication include hypercalcemia, hypercalciuria, confusion, polydipsia, polyuria, vomiting, and muscle weakness (6).

In spite of the growing concern for VDD, its prevalence in the population is still estimated to be between 24 and 40 % (3). The heterogeneity of recommendations for the prevention and treatment of VDD poses a great challenge for clinicians, but generally, 1000-2000 IU/day is recommended for adults to maintain adequate vitamin D levels (3, 31, 32). According to estimations, daily doses of 1000 IU vitamin D can be achieved by 5-15 minutes of skin exposure (on the face, arms, hands, and legs) to sunlight (1). However, the suggested amount of vitamin D also varies among different age and risk groups (3, 31). VDD can occur more frequently in newborns, children, and people with high skin pigmentation or who live in higher latitudes (4). Daily dietary intake of 400-600 IU vitamin D is recommended for infants and children to maintain proper bone health (28, 32). The risk of developing VDD also increases with advanced age, as elderly people tend to spend less time in the sunlight (13). Additionally, 7-dehydrocholesterol levels in the skin decline with aging, making the elderly more susceptible to developing VDD (1). Vitamin D supplementation is strongly advised for pregnant and lactating women, as well as for postmenopausal women who are at increased risk of osteoporosis due to lower estrogen production (26, 33). Higher body fat is associated with vitamin D sequestration; thus, it is more difficult for obese people to make vitamin D bioavailable (1). Patients with chronic hepatic or renal diseases, malabsorption syndromes, or those who take certain medications (e.g. glucocorticoids, antiretrovirals, anticonvulsants) are also exposed to an increased risk of deficiency (16). For obese people and individuals taking these medications, even 2-3 times more vitamin D intake is recommended than for the general population (31).

Sufficient levels of vitamin D are vital for a healthy skeleton, as the most substantial biological role of vitamin D is to enhance intestinal calcium absorption and maintain calcium homeostasis (6). The most common musculoskeletal health consequences of VDD are rickets, growth retardation, osteomalacia, osteopenia, osteoporosis, increased risk of fractures, and decreased muscle strength (34, 35). Interestingly, vitamin D has also been linked to numerous physiological and pathological mechanisms in relation to the

immune-, nervous, and cardiovascular systems, cancer development, and diabetes (12, 16, 36).

1.1.4. Impact on the vascular system

Several genes contain vitamin D response elements (e.g. renin, vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), interleukin-6, matrix metalloproteinase (MMP) 2, tissue inhibitors of MMP2 genes) which are involved in the regulation of the cardiovascular system, including for instance, the modulation of cell proliferation, differentiation and adhesion, oxidative stress, matrix homeostasis, and tissue mineralization (3, 7). Moreover, VDRs and 1-α-hydroxylase enzymes are localized in cardiovascular tissues and major cell types, such as endothelial cells, VSMCs, cardiomyocytes, immune cells, and platelets (5, 7).

Vitamin D can exert a considerable influence on vascular function (3). Nitric oxide (NO) is one of the most important mediators involved in vascular tone regulation and endothelium-dependent vasodilation (37, 38). Vitamin D can influence the bioavailability of NO within the endothelium by regulating the expression of eNOS and promoting its phosphorylation (3, 15). Vitamin D can also modulate vascular tone by the regulation of VSMC contractility *via* calcium influx and release of endothelial-derived vasoconstrictor mediators (17). Cyclooxygenase (COX) enzymes (COX-1, COX-2) participate in the production of endothelial-derived vasoactive factors from arachidonic acid, such as vasodilator prostacyclin and vasoconstrictor prostaglandin F2α or thromboxane A_2 (TXA_2) (39). Vitamin D has been reported to increase the production of prostacyclin in VSMCs (40) and may decrease COX-1 expression in endothelial cells (41), thereby altering the levels of vasoactive prostanoid mediators. Importantly, the unbalanced production of endothelial vasoactive mediators, which increases vasoconstrictor tone, can lead to endothelial dysfunction and arterial stiffening (15, 40). Endothelial dysfunction is also associated with increased levels of reactive oxygen species (ROS), proinflammatory and prothrombotic states, which may promote atherosclerotic plaque formation (3, 7, 35). Protecting from this, vitamin D can balance inflammatory responses by downregulating proinflammatory cytokines (e.g. interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor-α), upregulating anti-inflammatory cytokines (e.g. interleukin-4, interleukin-10), inhibiting prostaglandin signaling pathways (e.g. *via* COX-2 suppression, thromboxane-prostanoid receptor downregulation) and influencing the

function of immune cells (e.g. T cells, dendritic cells, macrophages) (7, 42). Vitamin D also decreases the expression of tissue factor, thrombospondin, and plasminogen activator inhibitor-1, but increases that of thrombomodulin, thus preventing thrombus formation (7). Additionally, vitamin D can reduce oxidative stress, for example, by inducing ROS-scavenging enzymes (e.g. catalase, superoxide dismutase, glutathione peroxidase), downregulating ROS-generating enzymes (e.g. nicotinamide adenine dinucleotide phosphate oxidases) and upregulating nuclear respiratory factor 2, which induces the expression of antioxidant enzymes (2, 15). Excessive oxidative stress may also lead to a decrease in NO bioavailability and thus, it further promotes endothelial dysfunction (15). Endothelial dysfunction has been implicated in the pathogenesis of several cardiovascular disorders (e.g. atherosclerosis, hypertension, peripheral arterial disease) (15), which indicates the importance of vitamin D in cardiovascular health prevention. In addition, VDD has been linked to endothelial senescence and arterial aging marked by reduced VSMC contractility and increased intima thickness and permeability (43, 44). Therefore, VDD may lead to accelerated vascular aging and, in turn, contribute to the advanced development of cardiovascular pathologies (43).

Furthermore, vitamin D can regulate cell proliferation, differentiation, and extracellular matrix homeostasis, which implies its role in angiogenesis and vascular remodeling (7, 45). In angiogenesis, VEGF is a key mediator, the expression and receptors of which are increased by vitamin D (46-48). VEGF promotes endothelial cell proliferation and migration and regulates vascular permeability (49). In addition, vitamin D can control VSMC proliferation and migration, which further supports that VDD may lead to adverse vascular remodeling (7, 15). Matrix metalloproteinases contribute to angiogenesis and vascular remodeling by degrading extracellular matrix proteins, and VDD has been reported to alter serum MMP levels and activity and the expression of their tissue inhibitors (3). For instance, tissue inhibitors of MMP-1, and MMP-3 are up-, while tissue inhibitors of MMP-2 and MMP-9 are downregulated (7). Additionally, VDD can alter the normal elasticity of the vessel wall, for example, by increasing its collagen and decreasing its elastin content (3, 50). Figure 1 summarizes the major impacts of VDD on the vessel wall.

Additionally, vitamin D regulates the renin-angiotensin-aldosterone system, which is responsible for the balance of vascular tone, peripheral resistance, and extracellular

fluid volume (34). Vitamin D inhibits renin expression in the kidneys so it can block the vasoconstrictor effect of angiotensin II produced, as well as the sodium-retaining effect of aldosterone (12). Therefore, vitamin D might be associated with an antihypertensive effect (7). Excessive activity of the renin-angiotensin-aldosterone system can result in high blood pressure and left ventricular hypertrophy (34), so VDD is linked to the development of hypertension and cardiac hypertrophy (3).

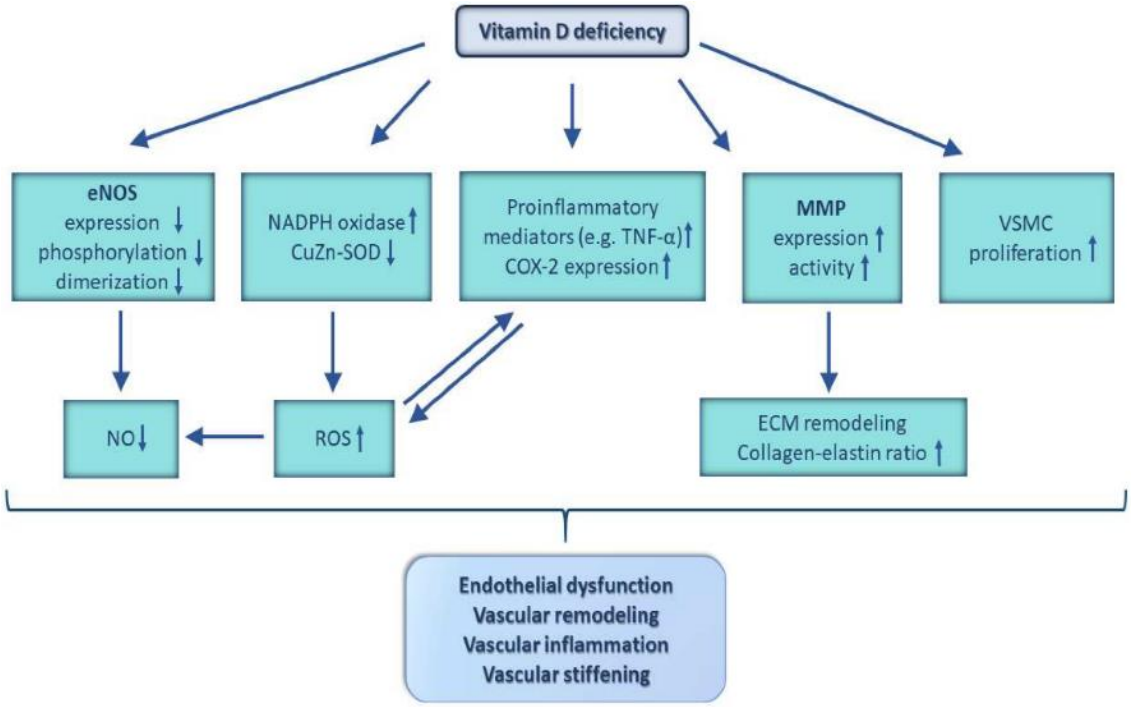

Figure 1. Adverse effects of vitamin D deficiency on the vessel wall. Figure adapted from Pál et al., which was published (and may be used) under the terms of CC-BY 4.0 (3). COX-2: cyclooxygenase 2, CuZn-SOD: copper-zinc superoxide dismutase, ECM: extracellular matrix, eNOS: endothelial nitric oxide synthase, MMP: matrix metalloproteinase, NADPH: nicotinamide adenine dinucleotide phosphate, NO: nitric oxide, ROS: reactive oxygen species, TNF-α: tumor necrosis factor-α, VSMC: vascular smooth muscle cell.

1.1.5. Vitamin D and cerebrovascular disorders

Cerebrovascular disorders, including ischemic stroke, belong to the leading causes of death and disability worldwide (2), and in the last decades, epidemiological studies have suggested an association between VDD and an elevated risk of developing stroke (2, 51). Moreover, vitamin D has been reported to increase the risk of hypertension, atherosclerosis, and diabetes, suggesting that VDD may indirectly increase the incidence

of cardio- and cerebrovascular diseases by these risk factors (3). In order to reveal the direct relationship between VDD and stroke, numerous studies addressed the seasonal and regional differences in stroke prevalence, investigating whether lower vitamin D levels during winter or higher latitudes affect stroke cases (51-53). Human studies examining the connection between serum vitamin D levels and stroke risk imply that people with higher vitamin D levels might be less prone to developing ischemic stroke (54, 55). Furthermore, epidemiological data suggest an unfavorable effect on post-stoke outcomes in VDD (2). For instance, low serum levels of vitamin D were associated with greater stroke severity, larger infarct volume, poorer outcomes, and higher incidence of subsequent cognitive impairment or recurrent stroke (15). Interestingly, severe VDD is especially common among people who have already had a stroke, suggesting that VDD may be attributed to age, malnutrition, or decreased sun exposure due to limited mobility rather than a consequence of stroke (53, 56). Additionally, the importance of vitamin D has been recognized in healthy aging with regard to preventing vascular function and age-related cognitive impairments (57). Overall, despite the fact that the results of observational studies support the connection between VDD and stroke, the results of Mendelian randomized trials are still inconsistent, so it remains in question whether vitamin D supplementation protects against stroke development and poorer outcomes (51).

Rodent studies also attempted to reveal the connection between VDD and cerebrovascular disorders and to discover the underlying mechanisms. Half of ischemic stroke cases occur due to atherosclerosis of large vessels (58), and it has been reported that VDD contributes to plaque formation and accelerates atherosclerosis by increased macrophage infiltration and accumulation, foam cell formation, endoplasmic reticulum stress, and by higher expression of adhesion molecules and proinflammatory cytokines (59-61). Experimental studies employing the most frequently used stroke model, middle cerebral artery (MCA) occlusion (58), reported that VDD can worsen the outcomes of occlusion, for example, by larger infarct volumes, more severe post-stroke behavioral impairments, lower neuroprotectant levels, altered inflammatory responses and increased blood-brain barrier dysfunction (62, 63). In contrast, Evans et al. reported that VDD in male mice did not affect infarct size and acute functional outcome after MCA occlusion (64). However, vitamin D supplementation before MCA occlusion attenuated infarct

volume size, neuronal injury, and inflammatory responses in rodents (65-67). Interestingly, vitamin D supplementation after MCA occlusion also improved cerebral perfusion and, therefore, alleviated neurological impairments in rats (68). Carotid artery stenosis accounts for 20% of all ischemic stroke cases (69), while complete occlusion of the carotid artery, mainly due to atherosclerosis, also accounts for 0.24-5% of stroke cases in humans (70, 71). We have recently demonstrated in male mice that the ablation of VDR signaling compromised the adaptation of the cerebrocortical microcirculation to unilateral common carotid artery occlusion (CAO), implying a functional impairment of cerebral vessels in VDD (72). Additionally, we discovered morphological alterations in leptomeningeal anastomoses in male VDR-mutant mice (72). In the case of primary artery blockade, leptomeningeal collaterals can provide an alternative way to deliver blood to the unsupplied territory (73); therefore, their morphology, condition, and number can considerably affect the outcome of an ischemic stroke (74).

Taken together, even though these findings suggest that VDD may influence the risk factors, development, and outcome of an ischemic stroke, controversial results exist, and the exact mechanisms have not been fully explored.

1.2. Estrogens and androgens

1.2.1. Sex steroid metabolism and signaling

The most important types of sex hormones are androgens (e.g. testosterone, dihydrotestosterone (DHT), androstenedione), estrogens (e.g. estradiol, estrone, estriol), and progestins (e.g. progesterone) (75, 76). Sex hormones are primarily produced in the gonads (i.e., ovaries, testes) and adrenal glands, but local synthesis also exists in extragonadal tissues, such as the brain and vasculature, making it possible for hormones to act as paracrine/autocrine factors (75, 77). All three classes are derived from the same precursor, cholesterol (78). Figure 2 demonstrates the pathway of steroidogenesis, including general localization and metabolites marked by the three major classes (77). Highlighting from the numerous steps (Figure 2), testosterone can be metabolized either by the 5α-reductase enzyme into its more potent form, DHT, or by the aromatase enzyme into the most active metabolite of estrogen, 17β-estradiol (76-78). In the circulatory system, most sex steroids are bound to sex hormone-binding globulins, but only the free, unbound fractions are biologically active (79). Their genomic effects are exerted through nuclear receptors - androgen receptor (AR), estrogen receptor (ER), and progesterone

receptor, on which hormones act as ligand-activated transcription factors (75). After ligand binding, the receptors homodimerize, translocate into the nucleus, and bind to androgen/estrogen response elements on target DNAs (80, 81). Two major forms of ER have been described: ERα and ERβ (75). The distribution of ER types is unequal across the body; ERα is rather present in the female reproductive system, whereas ERβ is more present, for example, in the cardiovascular system, central nervous system, colon, and prostate (81). Moreover, non-genomic, rapid actions of sex hormones, mediated by membrane receptors, ion channels, and enzymatic pathways, have been reported (75, 82). For example, an acute vasodilatory effect may involve the stimulation of eNOS phosphorylation, calcium channel inhibition, and potassium channel activation (83).

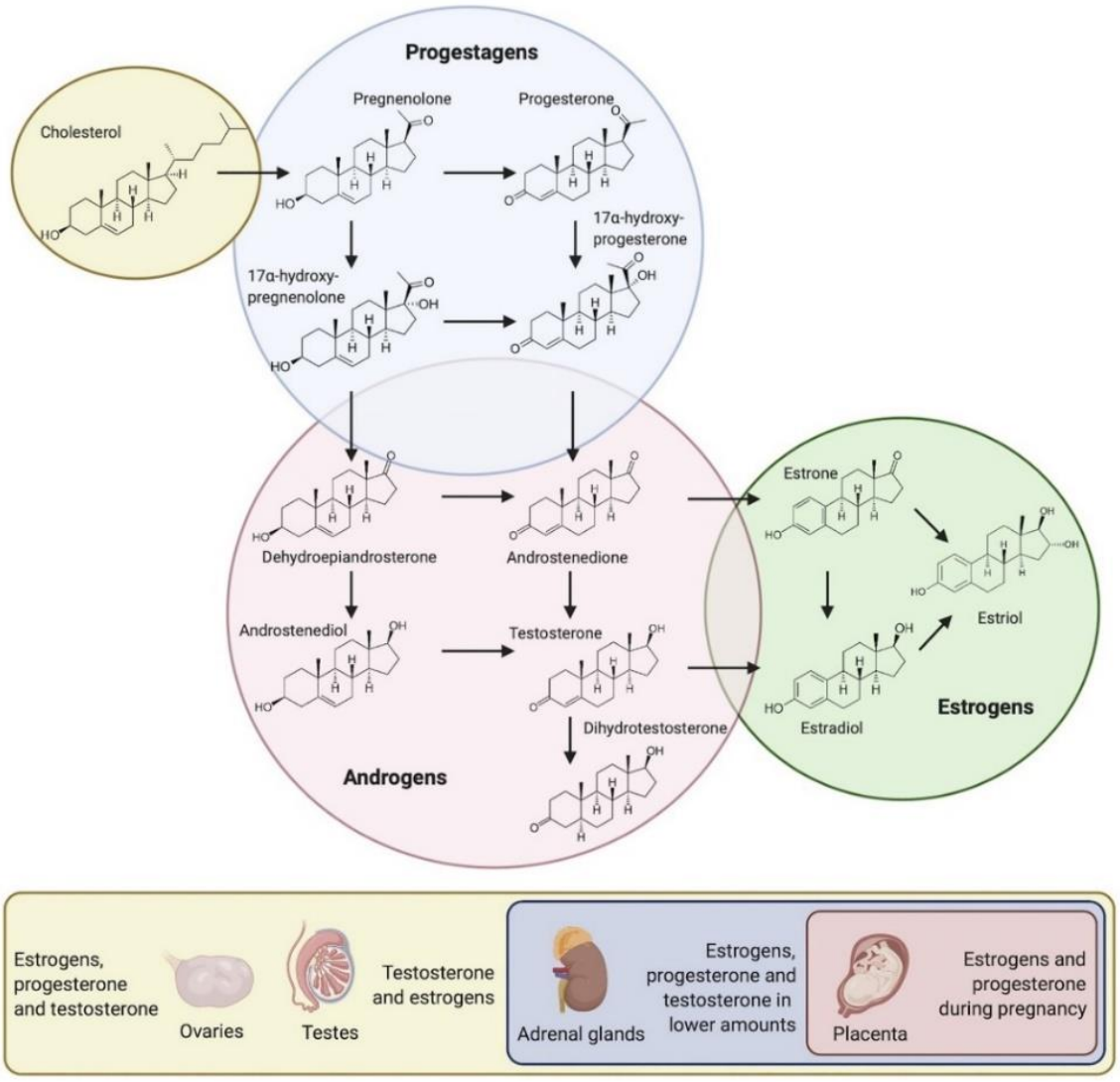

Figure 2. Metabolites of de novo steroidogenesis. Figure adapted from Cornejo et al., which was published (and may be used) under the terms of CC-BY 4.0 (77).

1.2.2. Cerebrovascular effects of estrogen and androgens

Sex steroids, particularly estrogen and androgens, appear to have a considerable impact on cerebral circulation (82). In contrast, progesterone receptor expression, as well as its impact on cerebral circulation, have yet to be fully revealed (75, 82). In rodents, ERα was found in the endothelium, ERβ in VSMCs, whereas AR is localized in both (84, 85). Although all receptors are found in male and female cerebral vessels, ERα expression has been reported to be enhanced by exposure to estrogen and, similarly, AR to androgens (85). Furthermore, gonadal metabolizing enzymes are expressed in cerebral vessels: 5α-reductase is present in both the endothelium and the smooth muscle layer, whereas the localization of aromatase is limited to the endothelium (75). Therefore, the local synthesis of the potent DHT and 17β-estradiol can result in different concentrations in the vessel wall from the circulating levels (75). Besides, sex hormones can act on other cells related to the neurovascular unit, such as astrocytes or neurons, and thus indirectly influence cerebral circulation (82). Figure 3 summarizes the main vascular effects of sex steroids mediated by AR and ERs.

Since sex hormone receptors and metabolizing enzymes are expressed in the cerebral vasculature, it allows them to alter the cerebrovascular tone, reactivity, and regulation of cerebral blood flow (80). Rodent studies suggested that female cerebral arteries dilate more *ex vivo* than those of males and ovariectomized females (86, 87). The reason might be the higher levels of estrogen, which is supported by the finding that estradiol replacement restored NO-mediated dilation in ovariectomized rodents to the extent observed in healthy females (84). Estrogen can stimulate eNOS expression and activity and consequently elevate NO levels (84). Moreover, estrogen increases the production of the potent dilator prostacyclin and shifts the prostanoid production towards vasodilation (88, 89). Interestingly, Deer et al. reported that while estrogen treatment increased vasodilation in small cerebral arteries of young ovariectomized rats, the treatment had the opposite effects in senescent females, suggesting an age-dependent beneficial effect of estrogen (90). The vasoactive effect of sex steroids on vascular function also appears to depend on the type of the examined vessel (91). Androgens have been reported to cause rapid vasodilation in the aorta and coronary arteries (83, 92); however, according to studies conducted in male rodents, cerebral vessels tend to constrict more with prolonged exposure to them (82, 84). For instance, the removal of testes resulted in a decreased

cerebrovascular tone, whereas testosterone or DHT replacement produced opposite effects (84). The reason might be that androgens increase the production of the potent vasoconstrictor TXA_2, suppress endothelium-derived hyperpolarizing factors, and consequently lead to an increased vascular tone (93, 94). Overall, estrogen and androgens seem to have opposing effects on the cerebrovascular tone (82), but the effect of androgens in females is less well-documented.

Sex steroids have also been reported to influence inflammatory processes and oxidative stress in the vasculature (75). Similarly to vascular tone regulation, the modulation of inflammatory responses by androgens and estrogen is the opposite (82). Estrogen prevents the overproduction of NO and prostaglandin E_2, characteristic of pathological conditions (e.g. ischemic stroke), by suppressing COX-2 and inducible NO synthase expression (84). Besides, estrogen decreases leukocyte adhesion, which effect may be reduced in ovariectomized rats (95, 96). Interestingly, it has been suggested that the anti-inflammatory effect of estrogen is similarly age-dependent, and that estrogen treatment is beneficial for young ovariectomized but not for naturally aged rats (97). Estrogen might also decrease the level of ROS, for instance, by regulating the activation and expression of superoxide dismutase 2 and nicotinamide adenine dinucleotide phosphate oxidases (75, 98). On the other hand, the effect of androgens varies depending on the type of metabolite and the physiological or pathophysiological state of the body (75). For example, testosterone promoted vascular inflammation in cerebral vessels by inducing COX-2 and inducible NO synthase expression after endotoxin treatment, but not under normal conditions (99). In contrast, DHT may promote inflammation under physiological conditions *via* the AR but might attenuate it under pathological conditions by conversion to 3β-androstanediol and activating ERβ (100, 101). Besides, DHT has potential antioxidant effects under pathological conditions (e.g. suppression of hypoxia-inducible factor-1 expression in hypoxia) (100). Moreover, testosterone may induce leukocyte migration and oxidative stress and cause VSMC apoptosis, thus potentially contributing to cardiovascular risk (92, 102, 103).

Additionally, sex steroids may contribute to vascular remodeling by influencing cell proliferation, migration, and differentiation (75). For instance, estrogen suppresses VSMC migration, while androgens may increase their proliferation (104, 105). Estrogen and androgens were reported to increase the expression of VEGF and its receptors,

suggesting a possible role in the regulation of angiogenesis (106-108). In addition to VEGF, the role of NO in angiogenesis has been suggested, the production of which can be influenced by estrogen (84, 108, 109). Accordingly, middle-aged ovariectomized rats had reduced capillary density in the frontal cortex, which was reversed by estrogen replacement (110). Besides, estrogen has been reported to improve vascular remodeling and long-term outcomes after cerebral ischemia in rats (111). Although the favorable effect of androgens on ischemia-induced angiogenesis has been suggested in mouse hindlimbs (112), rodent or human brain studies are lacking in the literature (75).

Taken together, sex steroids play a complex role in cerebrovascular function, with diverse effects regarding gender, age, and health conditions. Therefore, the exact impact of sex steroids on cerebral circulation in both genders requires further investigation.

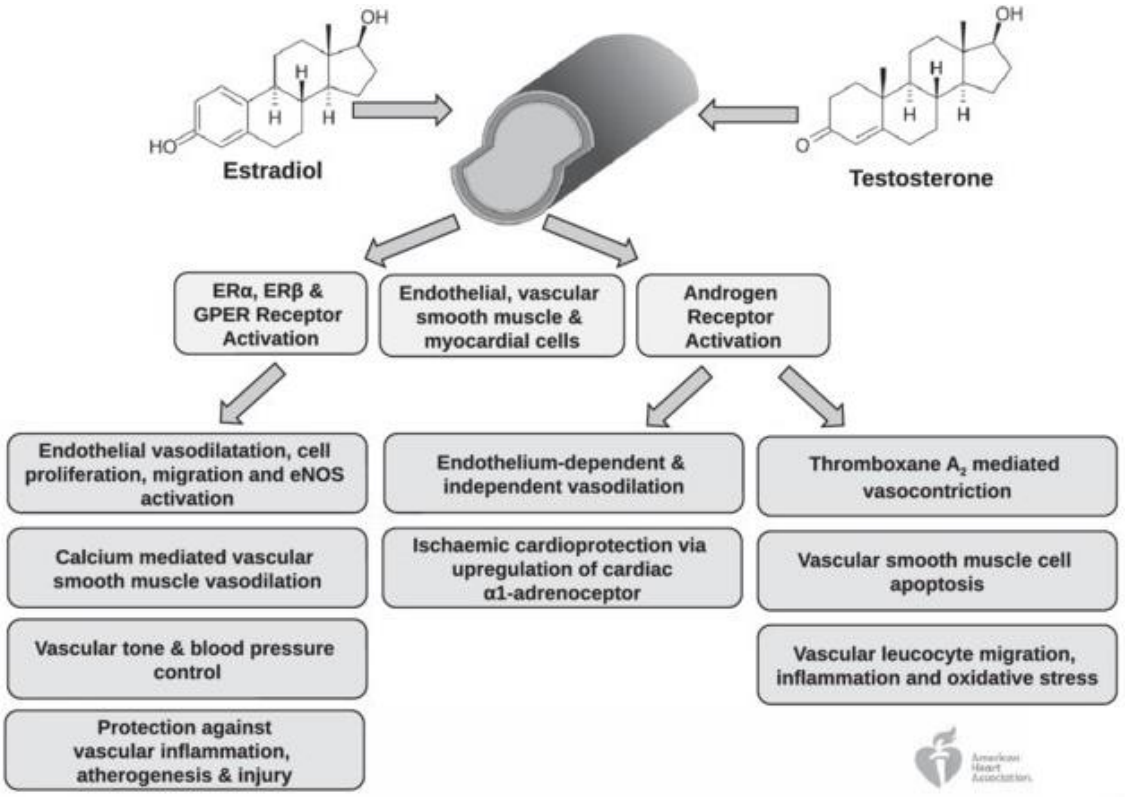

Figure 3. Summary of the vascular effects mediated by estrogen and androgen receptors. Figure adapted from Connely et al., which was published (and may be reused) under the terms of CC-BY 4.0 (92).

1.2.3. Gender differences in cerebrovascular disorders

The balanced production of sex steroids is vital for maintaining health in both genders (75). Epidemiological studies suggest gender differences in the incidence, prevalence, etiology, and outcome of ischemic stroke, which may be due in part to the different levels of sex hormones (particularly estrogen and testosterone) (86, 113). Men have been reported to have a higher incidence and mortality rate than women, but the difference diminishes with age (113, 114). This phenomenon may be related to hormonal changes due to menopause, as the ovaries no longer produce estrogen when reaching the age of menopause (115). Interestingly, animal studies suggested that estradiol treatment protects against brain injury (113), but also reported detrimental effects of estrogen treatment in aged female animals (90, 97). Similarly, human randomized clinical trials of hormone replacement therapy in postmenopausal women reported no benefit or even higher vascular risks (116). However, the increased risk in postmenopausal women has been related to age-dependent risk factors, such as higher blood pressure, dyslipidemia, or insulin resistance (117). In addition to declining estrogen levels, relatively higher androgen levels have also been associated with increased risk after menopause, but the results are inconsistent (118-121). During the reproductive age, the most common endocrine disorder in women with elevated androgen production is polycystic ovary syndrome (PCOS), with a worldwide prevalence of 6-20% (122). PCOS is associated with an increased risk of cardiovascular disorders due to insulin resistance, obesity, chronic inflammation, hypertension, endothelial dysfunction, and arterial stiffness (123-125).

In addition to the findings mentioned above on gender differences in vascular function, rodent studies also attempted to investigate sexual dimorphism in the outcome of ischemic stroke (126). For example, young female rats had smaller infarct sizes compared to age-matched males after MCA occlusion (127, 128). Interestingly, this phenomenon was not present when females were ovariectomized at an early age (127, 128), or in aged female rats (129). Moreover, estrogen treatment attenuated ischemic brain injury in young ovariectomized rats (128, 130, 131), reproductively senescent female rats (129, 131), and even male rats (132, 133). In contrast, testosterone replacement increased ischemic lesion size in castrated male rats (133). Although numerous studies indicate a neuroprotective role for estrogen, conflicting results suggest

that estrogen treatment has no or even an aggravating effect (134). Even though the effect of testosterone is well-described in males, the effect of androgens in females is less investigated and remains to be elucidated (80).

1.3. The importance of vitamin D and sex steroids in cerebrovascular disorders

Based on the aforementioned epidemiological and experimental studies, gender and vitamin D status may also impact the incidence and severity of cerebrovascular disorders. Even though women have been reported to have a lower incidence and mortality rate of cerebrovascular diseases than men, postmenopausal women and women in hyperandrogenic states are considered risk groups in terms of stroke, and their health condition is often accompanied by VDD (113, 123, 135, 136). The prevalence of VDD among postmenopausal women is approximately 80% (137), whereas 67-85% of PCOS women suffer from VDD (135). The importance of vitamin D supplementation to prevent bone fractures in postmenopausal women has been well established (136), but its importance may also extend to the prevention of cardiovascular events. However, epidemiological studies have not yet confirmed the preventive effect of vitamin D supplementation on cardiovascular events and its risk factors (137, 138). Nevertheless, VDD may exacerbate the cardiovascular consequences of conditions with altered hormonal status (i.e., menopause, hyperandrogenism). In hyperandrogenic women, low vitamin D levels were associated with worsening symptoms of PCOS, such as insulin resistance, irregular ovulation or menstrual cycle, obesity, hyperandrogenism, hirsutism, and a higher risk of cardiovascular disorders (135, 139). Additionally, it has been reported that high-dose vitamin D supplementation improved hyperinsulinemia, fertility, and hormonal status in PCOS, implying the importance of vitamin D in the clinical manifestation and treatment of PCOS (140).

Even though rodent studies have suggested that VDD exerts a deleterious effect on the cerebral vessels, only a few addressed the role of gender differences in the consequences of VDD. For instance, in anterior cerebral arteries isolated from male rats, VDD caused inward hypertrophic remodeling due to VSMC proliferation, endothelial dysfunction with impaired NO-mediated dilation, and altered vascular reactivity (141, 142). Interestingly, these alterations were not observed in vitamin-D-deficient female rats (142), but were manifested when VDD was combined with androgen excess (142, 143), suggesting gender differences and an important role of androgens in the vascular

consequences of VDD. Estrogen and vitamin D were reported to upregulate each other's receptors (144, 145), suggesting a possible interaction between them. Despite the fact that both menopause and VDD are associated with cerebrovascular disorders, the combined effect of VDD and estrogen deficiency on vascular function has not yet been extensively investigated. Furthermore, the exact sex-dependent vascular consequences of VDD, especially regarding sex hormone imbalance, have yet to be elucidated.

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2. Objectives

We have recently demonstrated the severe functional consequences of disrupted vitamin D signaling in the adaptation of cerebral microcirculation to unilateral CAO in male mice (72). Furthermore, we reported the significance of well-preserved pial collateral circulation in efficient cerebrocortical blood flow (CoBF) compensation following CAO (72). However, extracranial vessels, for instance, the contralateral carotid artery, may also play an important role in the adaptation to unilateral CAO. Therefore, our first goal was to examine the impact of VDR inactivity on the compensatory increase in blood flow in the contralateral carotid artery after unilateral CAO in male mice.

Considering the lower vulnerability of women to cerebrovascular disorders, we hypothesized that premenopausal healthy females may be more protected from the harmful effects of VDD compared to males, implying a protective role of estrogen in the cerebrovascular consequences of VDD. Therefore, we aimed to investigate the impact of VDR inactivity on the

- efficiency of the cerebrovascular adaptation to CAO,
- morphology of leptomeningeal collaterals and
- extracranial collateral circulation in healthy females.

Additionally, we hypothesized that estrogen deficiency or androgen excess in females might aggravate the cerebrovascular consequences of VDD. Therefore, our next goal was to investigate the efficiency of the cerebrovascular adaptation to CAO in ovariectomized and hyperandrogenic VDR-mutant female mice.

3. Methods

3.1. Experimental design

The experiments were performed in adult male and female mice carrying functionally inactive vitamin D receptors ($VDR^{\Delta/\Delta}$) and their wild-type (WT) littermates on a C57BL/6 genetic background, which were bred by intercrossing heterozygous animals (23). The animals were housed in a specific pathogen-free facility at constant temperature (19-22 °C) with a 12/12 light/dark cycle, and they had ad-libitum access to chow and water. To ensure normal calcium homeostasis, all animals were fed with a specific chow (so-called rescue diet) enriched with calcium (2%), phosphorus (1.25%), and lactose (20%) (8852-S010, SM Rescue Diet VDR KO, ssniff Spezialdiäten GmbH, Soest, Germany) (72). The mice were involved in the experiments at the age of 90-120 days. All procedures were approved by the National Scientific Ethical Committee on Animal Experimentation (PEI/001/2706-13/2014, approval date: 17 December 2014; PE/EA/00487-6/2021, approval date: 9 November 2021) and were conducted according to the guidelines of Hungarian Law of Animal Protection (XXVIII/1998). Table 1 summarizes the experimental design elaborated in the following.

Table 1. Experimental groups and the measurements conducted on them. The procedures were performed in female and male mice carrying functionally inactive vitamin D receptors ($VDR^{\Delta/\Delta}$) and wild-type (WT) littermates. Females were assigned to six experimental groups: intact controls ($VDR^{\Delta/\Delta}$, WT), ovariectomized (OVX-$VDR^{\Delta/\Delta}$, OVX-WT), and testosterone-treated (TT-$VDR^{\Delta/\Delta}$, TT-WT) groups. Vaginal cytology and cerebrocortical blood flow measurements were performed in all female groups, while the morphological analysis of leptomeningeal collaterals and carotid artery blood flow measurements were performed only in intact females. In the case of males ($VDR^{\Delta/\Delta}$♂, WT♂), only the results obtained from carotid artery blood flow measurements are presented in this thesis. Cerebrocortical blood flow measurements and morphological analysis were also performed in males (72), but those results are not included in this thesis.

Group	**WT**	**VDR $^{\Delta/\Delta}$**	**OVX-WT**	**OVX-VDR $^{\Delta/\Delta}$**	**TT-WT**	**TT-VDR $^{\Delta/\Delta}$**	**WT ♂**	**VDR$^{\Delta/\Delta}$♂**
Sex	♀	♀	♀	♀	♀	♀	♂	♂
Disrupted vitamin D signaling		✓		✓		✓		✓

Ovariectomy			✓	✓				
Testosterone treatment					✓	✓		
Vaginal cytology	✓	✓	✓	✓	✓	✓		
Cerebrocortical blood flow measurement	✓	✓	✓	✓	✓	✓		
Carotid artery blood flow measurement	✓	✓					✓	✓
Morphological analysis	✓	✓						

3.2. Ovariectomy and testosterone treatment

At three months of age, bilateral ovariectomy (OVX) was performed in 10 $VDR^{\Delta/\Delta}$ and 10 WT female mice under isoflurane (2%) anesthesia and sterile conditions (OVX-$VDR^{\Delta/\Delta}$ and OVX-WT groups). After the surgery, the mice were given analgesic (acetaminophen, 0.2 mg/g body weight, i.p., Paracetamol Kabi; Fresenius Kabi Hungary, Budapest, Hungary) and prophylactic antibiotic (ceftriaxone, 0.1 mg/g body weight, i.p., Ceftriaxon Kabi; Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) treatment, and their health status was monitored daily until the *in vivo* cerebrocortical blood flow measurements (see section 3.4.2.), which were performed five weeks after OVX. To induce androgen excess in female mice, 10 $VDR^{\Delta/\Delta}$ and 10 WT mice received daily transdermal testosterone treatment (0.033 mg/g body weight, Androgel 1%, Laboratories Besins International S.A., Paris, France) for five weeks (TT-$VDR^{\Delta/\Delta}$ and TT-WT groups) (143). The fur on a small area of the back was removed with a mouse razor under isoflurane (2%) anesthesia, which procedure was repeated when the fur grew back. To minimize blood level fluctuations, Androgel 1% was applied daily to the skin at the same time. The condition of these mice was also checked every day, and they did not show any signs of skin irritation. Until the experiments, these four groups were single-housed to ensure safe recovery from the surgery and to prevent fighting injuries. To assess weight gain due to OVX/testosterone treatment, body weight was measured before OVX or at the beginning of testosterone treatment and five weeks later (at the time of the *in*

vivo cerebrocortical blood flow measurements). The effectiveness of the testosterone treatment was examined by measuring testosterone concentrations in serum samples separated from arterial blood collected after the *in vivo* cerebrocortical blood flow measurements. Ultra-high performance liquid chromatography-tandem mass spectrometry was used to measure serum testosterone concentration (146). Due to the insufficient volume of plasma samples, estrogen concentration was not measured; thus, the success of the ovariectomy was validated by a suppressed estrus cycle (see section 3.3.) (147).

3.3. Vaginal cytology

Vaginal cytology was examined in intact, ovariectomized, and testosterone-treated $VDR^{\Delta/\Delta}$ and WT female mice for at least five consecutive days before conducting the *in vivo* cerebrocortical blood flow measurements (see section 3.4.2.). In the early mornings, vaginal smears were collected from awake animals by flushing the vaginal canal with 0.1 mL saline solution using syringes with blunt needles. Then, the stage of the estrus cycle was determined by evaluating the proportion of cornified epithelial cells, nucleated epithelial cells, and leucocytes in unstained samples under light microscopy (Zeiss Axio Imager.A1, Göttingen, Germany) (148, 149). Figure 4 shows the four phases of the estrus cycle (Figure 4A, B, C, D) and the proportion of cells in each stage (Figure 4E). In the ovariectomized groups, successful ovary removal was confirmed by suppressed estrus cycles (147), according to daily smear tests conducted for at least five consecutive days. The impact of testosterone treatment on the estrus cycle was examined, respectively. To avoid any hormonal biases, intact control and testosterone-treated mice were selected for the *in vivo* cerebrocortical blood flow measurements (see section 3.4.2.) in the diestrus phase, which can be accurately identified based on the high proportion of leukocytes (Figure 4D).

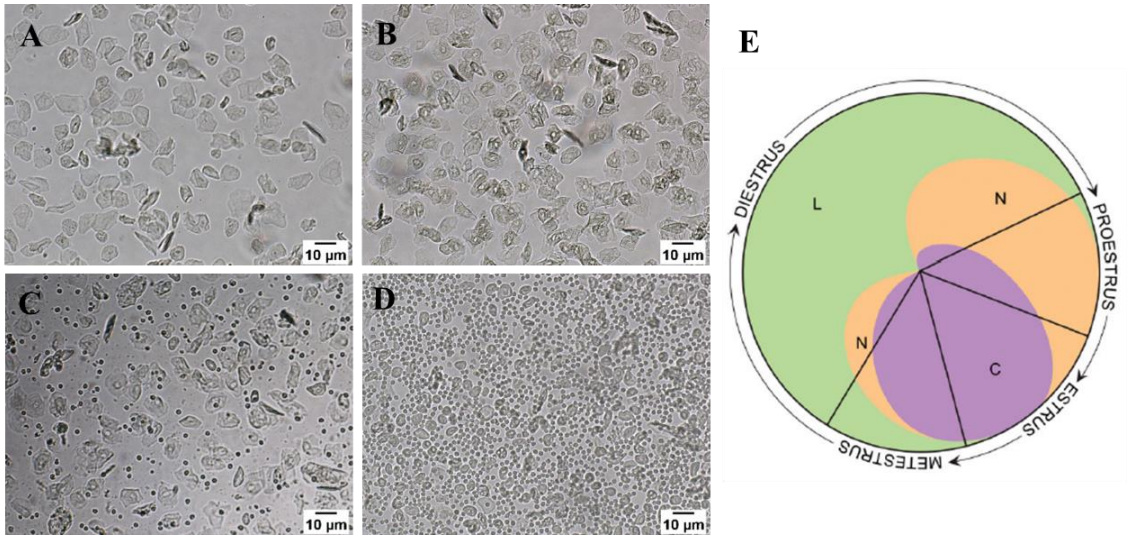

Figure 4. Four phases of the mouse estrus cycle under light microscopy in unstained samples: (A) proestrus, (B) estrus, (C) metestrus, (D) diestrus. (E) The relative proportion of cell types during the four stages of the estrus cycle. This figure (**E**) was adapted from Byers et al., which was published (and can be reused) under the terms of CC-BY 4.0 (149). C: cornified epithelial cells, L: leukocytes, N: nucleated epithelial cells.

3.4. *In vivo* measurements

3.4.1. Surgical procedures

Before the *in vivo* cerebrocortical (see section 3.4.2.) or carotid artery blood flow measurements (see section 3.4.3.), a surgical preparation was performed under a stereomicroscope (Wild M3Z, Heerbrugg, Switzerland). First, the body weight of animals was measured to determine the amount of drugs needed during the surgery and the *in vivo* measurements and to determine weight gain after OVX/testosterone treatment in the case of OVX-WT, OVX-VDR$^{\Delta/\Delta}$, TT-WT, TT-VDR$^{\Delta/\Delta}$ females. Then, the left femoral artery was cannulated under isoflurane (2%) anesthesia to measure arterial blood pressure during the *in vivo* experiments (150). After that, ketamine (100 μg/g body weight Calypsol; Gedeon Richter, Budapest, Hungary)-xylazine (10 μg/g body weight, CP-Xylazine; CP-Pharma, Burgdorf, Germany) was injected intraperitoneally as an anesthetic. To ensure free breathing, a cannula was inserted into the trachea. The last step of the surgery depended on which *in vivo* experiment was performed subsequently: for the cerebrocortical blood flow measurement (see section 3.4.2.), the left common carotid artery was carefully isolated, and a loose knot was placed around it for later occlusion, whereas for the carotid artery blood flow measurement (see section 3.4.3.), flow probes

were placed around both common carotid arteries and both external carotid arteries were ligated. Plantar nociception was frequently checked to maintain a sufficient depth of anesthesia, and when necessary, ketamine-xylazine was re-administered. The mice were kept on a heating pad controlled by a rectal thermometer to maintain body temperature at 37-38 ℃ (150).

3.4.2. Measurement of cerebrocortical blood flow using laser-speckle imaging

The *in vivo* cerebrocortical blood flow measurements were performed five weeks after ovariectomy or after the testosterone treatment (see section 3.2.). 10 $VDR^{\Delta/\Delta}$ and 10 WT intact mice were assigned to the control groups. CoBF changes induced by CAO were measured using laser-speckle imaging (PeriCam PSI; Perimed, Järfälla, Stockholm, Sweden) (150). After the surgery, the mouse was placed in a stereotaxic head holder, and a midline scalp incision was made to expose the skull, allowing the laser light to reach the cerebral cortex. Mean arterial blood pressure (MABP) was continuously recorded using the femoral artery cannula (MP100 System and AcqKnowledge 3.72 Software, Biopac Systems Inc, Goleta, CA, USA). Oxygen saturation, heart rate, and respiratory rate were continuously monitored using a pulse oximeter (MouseOx Plus, Starr Life Sciences Corp., Oakmont, PA, USA) placed on the right hindlimb. Then, atipamezole (1 μg/g ip.; Sigma-Aldrich, St. Louise, MO, USA), an α-2-antagonist, was injected to reverse the α-2-agonist effects of xylazine (150). After stabilizing the blood pressure and ensuring sufficient depth of anesthesia, baseline CoBF and MABP were recorded for one minute. Then, the loose knot around the left carotid artery was tightened to induce the occlusion, and CoBF changes were recorded for five minutes. Changes in CoBF were examined in three cerebrocortical regions in both hemispheres (with the hemisphere on the side of the occlusion referred to as ipsilateral, while the other hemisphere as contralateral): frontal, parietal, and temporal cortices (Figure 5) (150). CoBF changes were expressed as a percentage of the baseline reference value (100 %), which was calculated as the average of baseline CoBF during the one-minute recording before CAO. Two phases of the adaptation were analyzed separately: 0-30 s after the occlusion was considered the acute phase, and 31-300 s was the subacute phase. To assess the CoBF reductions quantitatively, the area over the curve (AOC) was calculated for each animal for both phases (i.e., acute, subacute) and all regions. At the end of the measurements, arterial blood gas tensions (pCO_2, pO_2), acid-base parameters (pH, bicarbonate

concentration), hematocrit, plasma ion concentrations (Ca^{2+}, Na^{+}, K^{+}, Cl^{-}), and oxygen saturation were determined by radiometric analysis (ABL80 FLEX Blood Gas Analyzer, Radiometer, Brønshøj, Denmark) following arterial blood sampling using the femoral artery cannula. Arterial blood samples were also collected for later serum testosterone concentration measurements (see section 3.2.). If systemic MABP was outside 70-120 mmHg, arterial O_2 saturation was lower than 90%, or CO_2 tension was outside 25-55 mmHg, the experiment was not evaluated. The complete occlusion of the carotid artery was confirmed under a stereomicroscope (150). One-one mouse was excluded from the OVX-WT and TT-$VDR^{\Delta/\Delta}$ groups, whereas two mice were from the $VDR^{\Delta/\Delta}$ group because of low MABP values or abnormal arterial blood gas tensions. Heart weight, brain weight, and tibial length were also measured after the experiments.

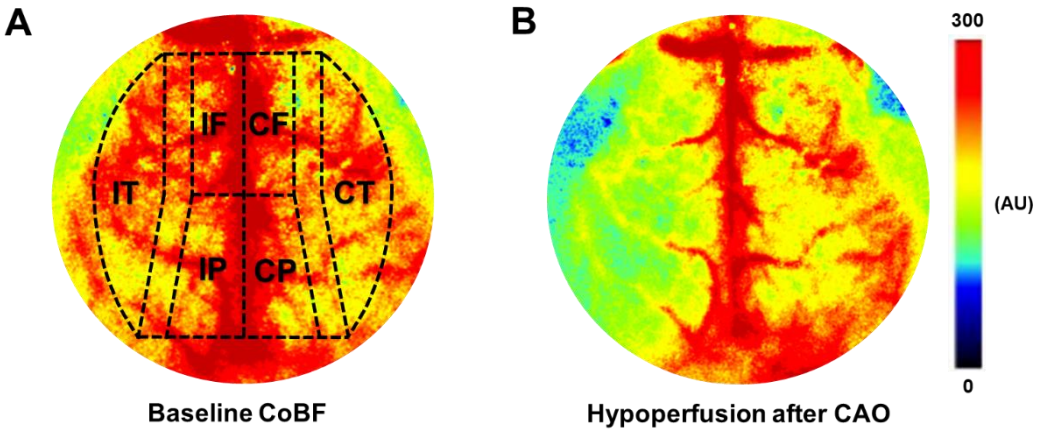

Figure 5. Cerebrocortical blood flow (CoBF) measurement with laser-speckle imaging. (**A**) Representative image of the cerebral cortex with baseline CoBF before carotid artery occlusion (CAO), indicating the localization of the regions of interest, where the CoBF changes were examined separately. Large vessels (outlined by red color - red color represents higher blood flow) were excluded from evaluation to analyze the microcirculation exclusively. (**B**) Representative image of the cerebral cortex with hypoperfusion (blue-green color) in the ipsilateral cortex after CAO. AU: arbitrary units, CF: frontal region of the contralateral hemisphere, CP: parietal region of the contralateral hemisphere, CT: temporal region of the contralateral hemisphere, IF: frontal region of the ipsilateral hemisphere, IP: parietal region of the ipsilateral hemisphere, IT: temporal region of the ipsilateral hemisphere.

3.4.3. Measurement of carotid artery blood flow using transit-time ultrasonic flowmeter

Carotid artery blood flow measurements were performed in 8 $VDR^{\Delta/\Delta}$ and 6 WT intact females and 6 $VDR^{\Delta/\Delta}{}_{\male}$ and 6 $WT_{\male}$ male mice (age: 90-120 days). Ultrasonic transit-time perivascular flow probes 0.5 PSB and TS420 flowmeter (Transonic System Inc, Ithaca, NY, USA) were used to measure carotid artery blood flow. Since the flow probes were not applicable to the internal carotid arteries, the external carotid arteries were ligated to measure the blood flow to the brain. Atipamezole was injected as described above (see section 3.4.2.), and after stabilization, baseline blood flow and MABP were recorded for one minute. Then, a vessel clip was placed distally to the flow probe to occlude the left common carotid artery (confirmed by measuring zero flow in the ipsilateral carotid artery), and the contralateral carotid artery blood flow and MABP were measured for five minutes. Vascular conductance was calculated as the ratio of blood flow (mL/min) and MABP (mmHg). Oxygen saturation, heart rate, and respiratory rate were continuously monitored using a pulse oximeter.

3.5. Morphological analysis of leptomeningeal collaterals

The cerebrocortical vasculature was visualized in 5 $VDR^{\Delta/\Delta}$ and 6 WT intact female mice (age: 90-120 days) under isoflurane anesthesia (2%) by transcardial perfusion with 10 mL of heparinized saline solution (10 IU/mL) followed by 2 mL of a mixture of black ink (Koh-I-Noor Hardmuth, Cescké Budejovice, Czech Republic), endorsing ink (Interaction-Connect, Gent, Belgium) and distilled water in the ratio of 6:1:6. Then, the brains were removed after decapitation, and fixed with formaldehyde solution (4%) for at least 24 hours. ImageJ software (ImageJ 1.5 NIH, Bethesda, MD, USA) was used to evaluate the morphology of leptomeningeal collaterals that connect the branches of the anterior cerebral artery (ACA) and the middle cerebral artery (MCA) in digital images captured by a digital camera attached to the microscope (Leica MC 190 HD and Leica M80, Leica Microsystems, Wetzlar, Germany). The number of collaterals, tortuosity index (the ratio of vessel curve length and linear distance between the two ends of the vessel), and the distance between the anastomotic line (a line connecting the half-distance points between the nearest branching points of the ACA and MCA branches) and the midline were determined in each hemisphere.

3.6. Statistical analysis

The normal distribution of the data was confirmed by the Shapiro-Wilk test, and the data are presented as mean ± SEM. If the distribution of the data was not normal, the data are presented as median and interquartile range. The statistical significance of results obtained from the *in vivo* experiments was determined using two-way ANOVA followed by Bonferroni's or Tukey's post hoc test (depending on the number of variables). ANOVA was carried out after data transformation when the distribution of data was not normal. The significance of weight gain was assessed by the Student's paired t-test or Wilcoxon test. In the morphological analysis, p-values were calculated by the Student's unpaired t-test. GraphPad Prism software (v.8.0, GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis, and $p<0.05$ was considered a statistically significant difference.

4. Results

4.1. Anatomical and physiological features

4.1.1. Anatomical traits of female mice

Body weight, heart weight, brain weight, and tibia length were measured in female mice to investigate the impact of VDR inactivity and hormonal changes on these general anatomical parameters. None of these parameters were different between intact $VDR^{\Delta/\Delta}$ and WT females; however, intact $VDR^{\Delta/\Delta}$ mice had shorter tibia lengths than OVX-WT and TT-WT mice (Table 2). TT-WT mice had significantly higher body weight than intact females ($VDR^{\Delta/\Delta}$, WT), OVX-$VDR^{\Delta/\Delta}$, and TT-$VDR^{\Delta/\Delta}$ mice (Table 2).

Table 2. Anatomical traits of intact, ovariectomized (OVX), and testosterone-treated (TT) vitamin D receptor-mutant ($VDR^{\Delta/\Delta}$) and wild-type (WT) female mice at the end of the experiments. Significantly higher body weight was observed in the TT-WT group compared to the intact WT ($^{*}p<0.05$), $VDR^{\Delta/\Delta}$ ($^{**}p<0.01$), OVX-$VDR^{\Delta/\Delta}$ ($^{\dagger}p<0.05$) and TT-$VDR^{\Delta/\Delta}$ ($^{\#}p<0.05$) groups. The tibia length of $VDR^{\Delta/\Delta}$ females was significantly shorter compared to OVX-WT ($^{**}p<0.01$) and TT-WT ($^{\dagger}p<0.05$) mice. No differences were found among the experimental groups regarding the other parameters. Data are presented as mean ± SEM or median and interquartile range, n=10 in all groups, two-way ANOVA followed by Tukey's post hoc test.

Parameter	WT	$VDR^{\Delta/\Delta}$	OVX-WT	OVX-$VDR^{\Delta/\Delta}$	TT-WT	TT-$VDR^{\Delta/\Delta}$
Body weight (g)	22.10 ± 0.18	20.88 ± 0.44	23.56 ± 0.42	21.82 ± 0.30	25.34 ± 1.40*, **, †, #	22.22 ± 0.82
Heart weight (g)	0.13 (0.12-0.14)	0.12 (0.12-0.14)	0.13 (0.12-0.13)	0.12 (0.11-0.12)	0.13 (0.12-0.14)	0.12 (0.11-0.12)
Heart weight / Body weight (%)	0.58 ± 0.02	0.60 ± 0.03	0.53 ± 0.02	0.53 ± 0.01	0.55 ± 0.05	0.58 ± 0.03
Tibia length (cm)	1.66 ± 0.03	1.59 ± 0.04**, †	1.76 ± 0.03	1.67 ± 0.03	1.75 ± 0.03	1.68 ± 0.03
Brain weight (g)	0.442 ± 0.005	0.438 ± 0.003	0.441 ± 0.008	0.442 ± 0.009	0.451 ± 0.006	0.440 ± 0.006

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Additionally, both ovariectomy and the five-week testosterone treatment caused a significant increase in body weight compared to the body weights measured before OVX/testosterone treatment (Figure 6).

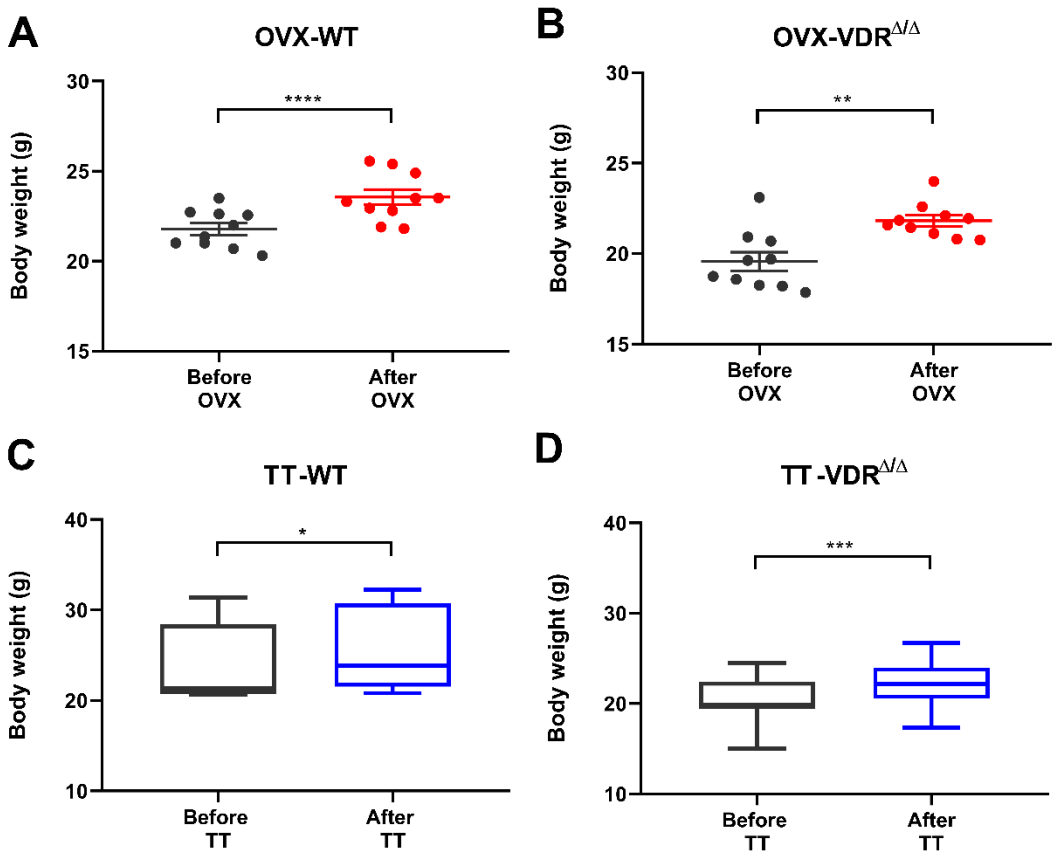

Figure 6. Weight gain of vitamin D receptor-mutant (VDR$^{\Delta/\Delta}$) and wild-type (WT) female mice after ovariectomy (OVX) or testosterone treatment (TT). Bilateral ovariectomy resulted in a significant weight gain five weeks after the surgery in OVX-VDR$^{\Delta/\Delta}$ and OVX-WT mice. Similarly, the five-week-long testosterone treatment significantly increased the body weight of TT-VDR$^{\Delta/\Delta}$ and TT-WT females. Data are presented as mean ± SEM or median and interquartile range, n=10 in all groups, paired t-test or Wilcoxon test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

4.1.2. Validation of testosterone treatment and ovariectomy

Testosterone concentration was measured in serum samples separated from arterial blood collected after the *in vivo* experiments. Although testosterone concentrations were below the detection limit (0.05 ng/mL) in the intact control (WT, $VDR^{\Delta/\Delta}$) and ovariectomized groups (OVX-WT, OVX-$VDR^{\Delta/\Delta}$), testosterone concentrations were well above the detection limit in both testosterone-treated groups, indicating the success of the transdermal treatment to induce androgen excess. Testosterone concentrations measured in the TT-WT (1.51 ± 0.25 ng/mL) and TT-$VDR^{\Delta/\Delta}$ (1.06 ± 0.18 ng/mL) groups were not different from each other (*n*=8-8, unpaired t-test).

The estrus cycle of females was also determined for five consecutive days before the *in vivo* experiments to examine the impact of ovariectomy on estrus cyclicity. Ovariectomized groups showed estrus acyclicity five weeks after the removal of the ovaries, whereas intact females had normal estrus cycles.

4.1.3. Morphology of leptomeningeal collaterals in intact females

In the event of a primary artery blockade, leptomeningeal collaterals can provide an alternative route to deliver blood to unsupplied areas (73). Therefore, the number and morphology of collaterals can considerably affect the outcome (74). Previously, we discovered morphological alterations of pial anastomoses in male mice carrying functionally inactive VDRs (72). To investigate whether high estrogen levels in females can protect from unfavorable morphological changes due to non-functioning VDRs, we examined the number and tortuosity of collaterals between the branches of MCA and ACA, and determined the distance of the anastomotic line from the midline, which can be used to differentiate the cortical territories supplied by the MCA or ACA (Figure 7). In $VDR^{\Delta/\Delta}$ females, the number of MCA-to-ACA collaterals was significantly smaller compared to WT mice (Figure 7C). However, the tortuosity (Figure 7D) and localization of the collaterals (Figure 7E) were not different between the groups.

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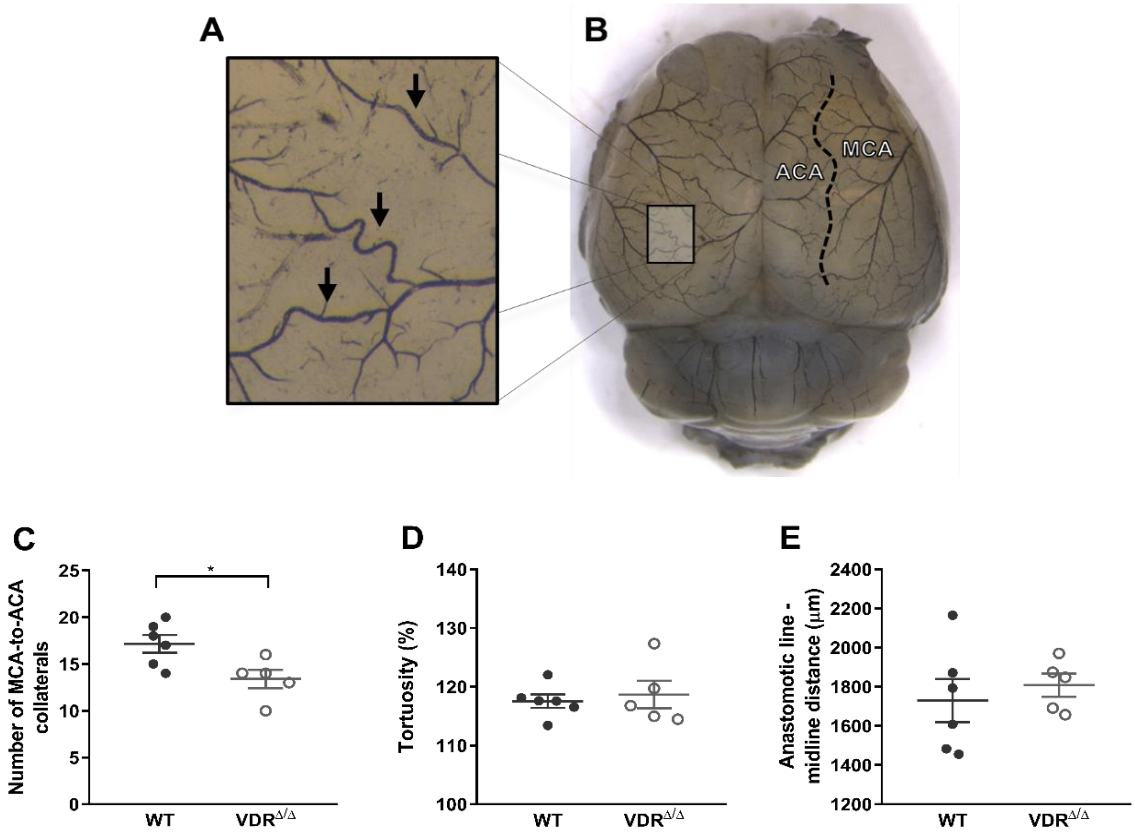

Figure 7. Morphological analysis of leptomeningeal collaterals in intact vitamin D receptor-mutant (VDR$^{\Delta/\Delta}$) and wild-type (WT) female mice. (**B**) Representative image of a mouse brain with visualized vessels. The dashed line in the right hemisphere represents the line of anastomosis through which an anastomotic connection can occur between areas supplied by the middle cerebral artery (MCA) and the anterior cerebral artery (ACA). (**A**) In the magnified image, the arrows show the collaterals connecting the branches of the MCA and the ACA, the number and tortuosity of which were examined. The number of MCA-to-ACA collaterals was significantly smaller in VDR$^{\Delta/\Delta}$ females compared to WT mice (**C**), but no difference was found in their tortuosity (**D**). The anastomotic line-midline distance in VDR$^{\Delta/\Delta}$ females did not differ from WT mice, indicating that the localization of the anastomotic line was not influenced by VDR inactivity (**E**). Data are presented as mean ± SEM, n(WT)=6, n(VDR$^{\Delta/\Delta}$)=5, where n refers to the number of brains analyzed, Student's unpaired t-test (*$p<0.05$).

4.2. *In vivo* measurements

4.2.1. Impact of vitamin D receptor deficiency and sex on the extracranial circulation

The contralateral carotid artery also plays a significant role in the compensation of cerebrocortical blood flow after CAO by providing blood supply to the hypoperfused area from the unaffected hemisphere (150, 151). Therefore, we aimed to investigate the increase in blood flow in the intact contralateral carotid artery using a transit-time ultrasonic flowmeter in male ($VDR^{\Delta/\Delta}$♂, WT♂) and female ($VDR^{\Delta/\Delta}$, WT) mice. MABP was continuously measured using a femoral artery cannula to calculate vascular conductance. In male mice ($VDR^{\Delta/\Delta}$♂, WT♂), the blood flow similarly increased in the intact carotid artery after CAO in both groups (Figure 8A). Vascular conductance was also enhanced (Figure 8C), as no major changes were registered in MABP after CAO (Figure 8B). No significant differences were found between the male groups in these parameters (Figure 8), which suggests that the cerebrocortical compensation following CAO was not compromised by altered adaptation of the contralateral carotid artery due to disrupted vitamin D signaling.

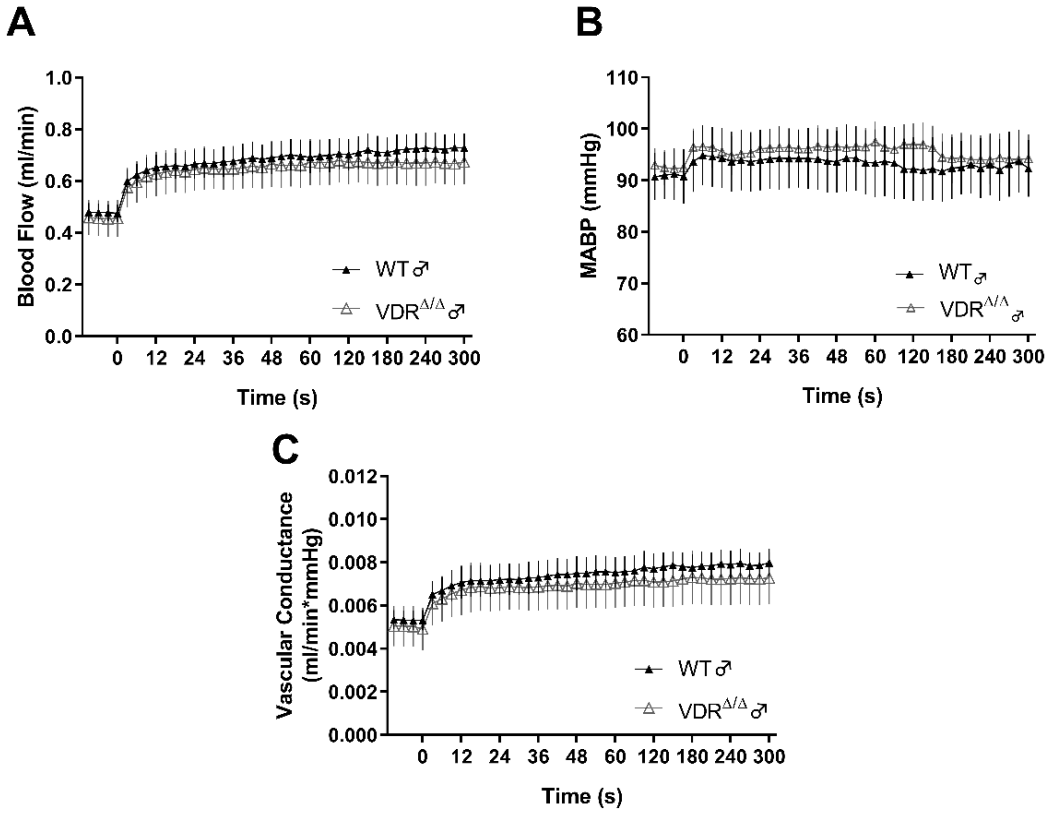

Figure 8. Changes of blood flow, mean arterial blood pressure (MABP), and vascular conductance in the intact contralateral carotid artery after unilateral

carotid artery occlusion (CAO) in male mice carrying functionally inactive vitamin D receptors (VDR$^{\Delta/\Delta}$♂) and wild-type (WT♂) littermates. The zero point on the time scale indicates the moment of CAO. CAO caused only a minor increase in MABP in both groups (**B**) but caused a more pronounced increase in blood flow in the contralateral carotid artery (**A**). Vascular conductance (calculated as blood flow divided by MABP) was enhanced following CAO (**C**). None of the examined parameters was different between the experimental groups. Data are presented as mean ± SEM, n=6 in both groups, two-way ANOVA followed by Bonferroni's post hoc test.

Similar observations were made in female mice (VDR$^{\Delta/\Delta}$, WT) when examining these parameters. Contralateral carotid artery blood flow similarly increased after CAO in both groups (Figure 9A). MABP was slightly affected by CAO (Figure 9B); thus, vascular conductance was increased (Figure 9C). No significant differences were found between the groups in these parameters (Figure 9), indicating that VDR inactivity did not alter the adaptation of the contralateral carotid artery following CAO in females.

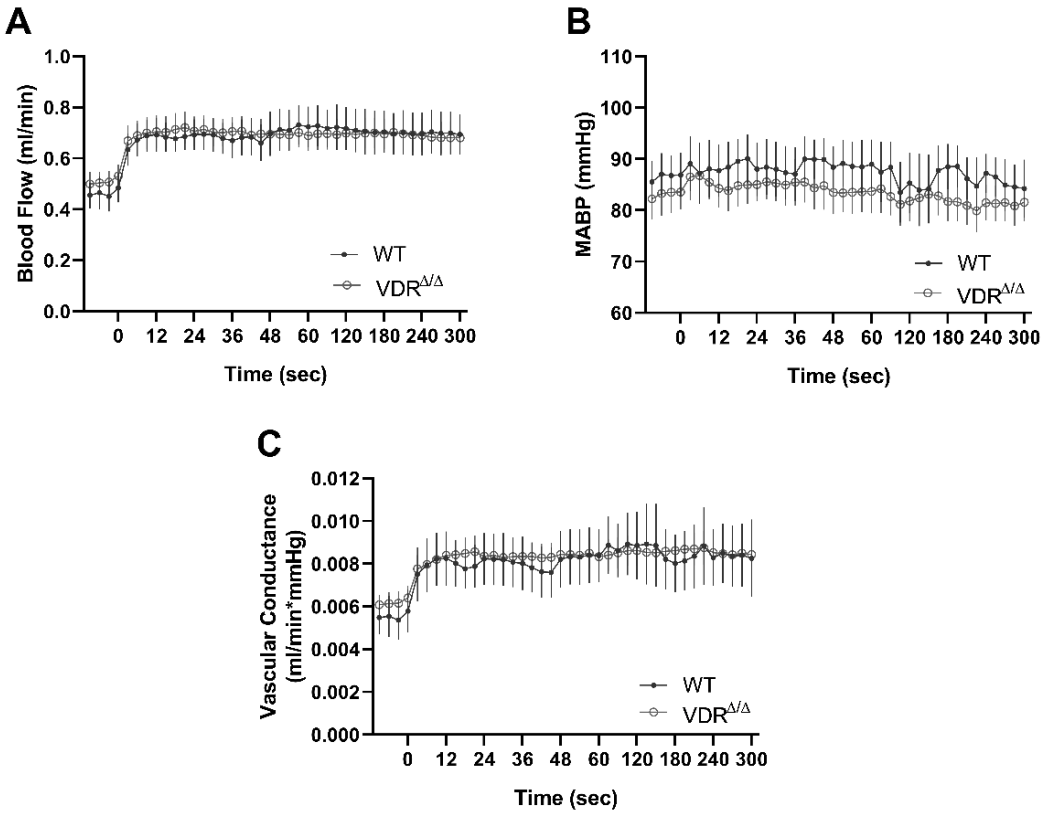

Figure 9. Changes of blood flow, mean arterial blood pressure (MABP), and vascular conductance in the intact contralateral carotid artery after unilateral carotid artery occlusion (CAO) in female mice carrying functionally inactive

vitamin D receptors ($VDR^{\Delta/\Delta}$) and wild-type (WT) littermates. The zero point on the time scale indicates the moment of CAO. CAO caused a pronounced increase in contralateral carotid artery blood flow in both groups (**A**), but MABP was less affected (**B**). Vascular conductance (calculated as blood flow divided by MABP) was enhanced following CAO (**C**). None of the examined parameters was different between the experimental groups. Data are presented as mean ± SEM, n(WT)=6, n($VDR^{\Delta/\Delta}$)=8, two-way ANOVA followed by Bonferroni's post hoc test.

Heart rate, respiratory rate, and O_2 saturation were also continuously monitored during the *in vivo* carotid artery blood flow measurements, and no significant differences were found in these parameters among the groups (Table 3).

Table 3. **Heart rate, respiratory rate, and oxygen saturation in males and females.** Heart rate, respiratory rate, and O_2 saturation were recorded during the *in vivo* measurements of carotid artery blood flow in male vitamin D receptor-mutant ($VDR^{\Delta/\Delta}$♂) and wild-type (WT♂) mice, as well as in female vitamin D receptor-mutant ($VDR^{\Delta/\Delta}$) and wild-type (WT) mice. None of the parameters was different between these experimental groups. Data are presented as mean ± SEM or median and interquartile range, n(WT♂)=6, n($VDR^{\Delta/\Delta}$♂)=6, n(WT)=6, n($VDR^{\Delta/\Delta}$)=8, two-way ANOVA followed by Tukey's post hoc test.

Parameter	**WT♂**	**$VDR^{\Delta/\Delta}$♂**	**WT**	**$VDR^{\Delta/\Delta}$**
Heart rate (1/min)	386.1 ± 14.4	387.9 ± 24.1	391.5 ± 29.7	362.4 ± 25.5
Respiratory rate (1/min)	115.9 ± 23.3	103.0 ± 22.8	79.5 ± 14.0	78.8 ± 3.7
O_2 saturation (%)	95.7 ± 0.8	93.4 ± 2.2	92.4 ± 1.5	95.5 ± 1.1

4.2.2. Effects of vitamin D receptor deficiency and hormonal changes on the cerebrocortical blood flow changes after carotid artery occlusion in females

4.2.2.1. *In vivo* blood pressure measurements

Systemic mean arterial blood pressure was continuously recorded during the *in vivo* cerebrocortical blood flow measurements. The one-minute long recording before CAO was considered baseline MABP, and the changes in MABP were recorded for five minutes after CAO. Average MAPB values were calculated for each minute for all mice and then statistically assessed (Table 4). CAO only caused a slight increase in MABP in all groups, and no significant differences were found in MABP values among the experimental groups (Table 4). In addition, the MABP of all groups was in the physiological range and above the lower limit of cerebral autoregulation during the

experiments (Table 4). Therefore, we can exclude the possibility that blood pressure altered the cerebrovascular autoregulatory capacity in any of the experimental groups.

Table 4. Mean arterial blood pressure (MABP) values during the *in vivo* cerebrocortical blood flow measurements. MABP was monitored in intact, ovariectomized (OVX), and testosterone-treated (TT) vitamin D receptor-mutant ($VDR^{\Delta/\Delta}$) and wild-type (WT) female mice. Baseline MABP was defined as the mean of the one-minute-long recording before carotid artery occlusion (CAO). CAO was performed at time point zero, and MABP was monitored for five more minutes. Average MABP values were calculated for all minutes for each mouse and then compared among the experimental groups. MABP values of all groups were in the physiological range and within the cerebral autoregulation range during the experiments. No significant differences were found among the experimental groups. Data are presented as mean ± SEM, n(WT)=10, n($VDR^{\Delta/\Delta}$)=8, n(OVX-WT)=9, n(OVX-$VDR^{\Delta/\Delta}$)=10, n(TT-WT)=10, n(TT-$VDR^{\Delta/\Delta}$)=9, two-way ANOVA followed by Tukey's post hoc test.

MABP (mmHg)	**WT**	**$VDR^{\Delta/\Delta}$**	**OVX-WT**	**OVX-$VDR^{\Delta/\Delta}$**	**TT-WT**	**TT-$VDR^{\Delta/\Delta}$**
Baseline	79.38 ± 3.11	76.12 ± 2.30	75.72 ± 3.34	70.54 ± 2.23	75.91 ± 2.07	75.72 ± 2.70
0-60 s	83.17 ± 3.36	80.38 ± 2.72	81.21 ± 3.36	74.91 ± 1.63	83.04 ± 1.95	82.51 ± 2.90
61-120 s	82.72 ± 3.62	79.02 ± 2.18	80.53 ± 3.00	74.94 ± 2.34	83.34 ± 2.11	80.62 ± 3.28
121-180 s	83.08 ± 3.55	77.11 ± 2.58	79.16 ± 2.98	71.59 ± 2.18	81.97 ± 2.21	78.49 ± 2.80
181-240 s	83.09 ± 3.53	76.87 ± 2.56	78.88 ± 3.11	71.28 ± 2.20	82.03 ± 2.28	78.72 ± 3.18
241-300 s	83.72 ± 3.56	76.11 ± 2.53	78.37 ± 3.04	71.57 ± 2.10	81.60 ± 2.07	79.21 ± 3.50

4.2.2.2. Analysis of blood gas, acid-base parameters, and plasma ion concentrations

Arterial blood gas (pCO_2, pO_2), acid-base parameters (pH, bicarbonate concentration), hematocrit, plasma ion concentrations (Na^+, K^+, Ca^{2+}, Cl^-), and O_2 saturation were analyzed in the arterial blood samples at the end of each *in vivo* cerebrocortical blood flow measurement. None of these parameters was different among the experimental groups (Table 5). Calcium ion concentrations were similar in VDR-deficient and WT mice, which indicates that the rescue diet normalized calcium homeostasis in VDR-deficient mice (23). We can exclude the possibility that arterial

blood gas tensions altered the cerebrovascular autoregulatory capacity in any of the experimental groups, as all parameters were within the physiological range (Table 5).

Table 5. Arterial blood gas, acid-base parameters, and plasma ion concentrations. Arterial blood gas, acid-base parameters, and plasma ion concentrations were determined after the *in vivo* cerebrocortical blood flow measurements in intact, ovariectomized (OVX) and testosterone-treated (TT) vitamin D receptor-mutant ($VDR^{\Delta/\Delta}$) and wild-type (WT) female mice. No significant differences were found in these parameters among the experimental groups. Data are presented as mean ± SEM or median and interquartile range, n(WT)=10, n($VDR^{\Delta/\Delta}$)=8, n(OVX-WT)=9, n(OVX-$VDR^{\Delta/\Delta}$)=10, n(TT-WT)=10,n(TT-$VDR^{\Delta/\Delta}$)=9, two-way ANOVA followed by Tukey's post hoc test.

Parameter	WT	$VDR^{\Delta/\Delta}$	OVX-WT	OVX-$VDR^{\Delta/\Delta}$	TT-WT	TT-$VDR^{\Delta/\Delta}$
pH	7.29 ± 0.02	7.27 ± 0.01	7.30 ± 0.01	7.28 ± 0.02	7.26 ± 0.02	7.27 ± 0.02
pCO_2 (mmHg)	40.35 (35.30-47.03)	38.20 (35.28-47.38)	45.00 (36.65-46.90)	35.60 (31.43-45.93)	48.85 (31.70-50.85)	43.60 (38.60-45.90)
pO_2 (mmHg)	96.50 ± 3.65	93.00 ± 4.56	94.22 ± 2.41	99.00 ± 4.81	93.4 ± 3.35	98.44 ± 3.36
Hematocrit (%)	42.10 ± 1.14	41.0 ± 1.09	39.89 ± 0.98	39.70 ± 0.60	39.30 ± 0.63	38.44 ± 0.58
cNa^+ (mmol/L)	155.60 ± 1.19	156.10 ± 1.04	157.90 ± 1.20	157.90 ± 1.20	156.50 ± 0.82	154.80 ± 0.76
cK^+ (mmol/L)	4.33 ± 0.13	4.35 ± 0.12	4.47 ± 0.06	4.29 ± 0.14	4.27 ± 0.10	4.34 ± 0.15
cCa^{2+} (mmol/L)	1.28 ± 0.02	1.22 ± 0.02	1.27 ± 0.03	1.25 ± 0.02	1.29 ± 0.01	1.27 ± 0.02
cCl^- (mmol/L)	116.20 ± 1.87	115.80 ± 0.94	116.00 ± 1.23	115.40 ± 1.51	115.20 ± 0.92	115.10 ± 0.63
$cHCO_3^-$ (mmol/L)	18.83 ± 0.82	17.90 ± 0.76	19.09 ± 0.96	18.22 ± 0.73	19.07 ± 0.28	18.84 ± 0.72
O_2 saturation (%)	97.28 ± 0.51	96.20 ± 0.68	97.33 ± 0.34	97.25 ± 0.50	96.04 ± 0.62	97.17 ± 0.38

4.2.2.3. Regional cerebrocortical blood flow changes after carotid artery occlusion

The blood supply to the frontal region is predominantly provided by the azygous anterior cerebral artery (AACA), which is supplied from both sides of the circle of Willis (150). Figure 10A shows the CoBF changes after CAO in the frontal region of the ipsilateral hemisphere (which is on the side of the occlusion), while Figure 10B in the contralateral hemisphere. According to the calculated AOC values, no significant differences were discovered between the groups in the acute phase in the two hemispheres (Figure 10C, D), which indicated a similar degree of hypoperfusion in all groups. However, a considerable difference was found in the subacute phase as the AOC values were higher in the TT-VDR$^{\Delta/\Delta}$ group compared to intact females (VDR$^{\Delta/\Delta}$, WT) and OVX-WT mice, indicating that TT-VDR$^{\Delta/\Delta}$ mice suffered a significantly prolonged reduction in blood flow in the ipsilateral hemisphere (Figure 10E). On the contrary, the subacute phase of the contralateral hemisphere was less impacted, and no significant differences were observed among the groups in the calculated AOCs (Figure 10F).

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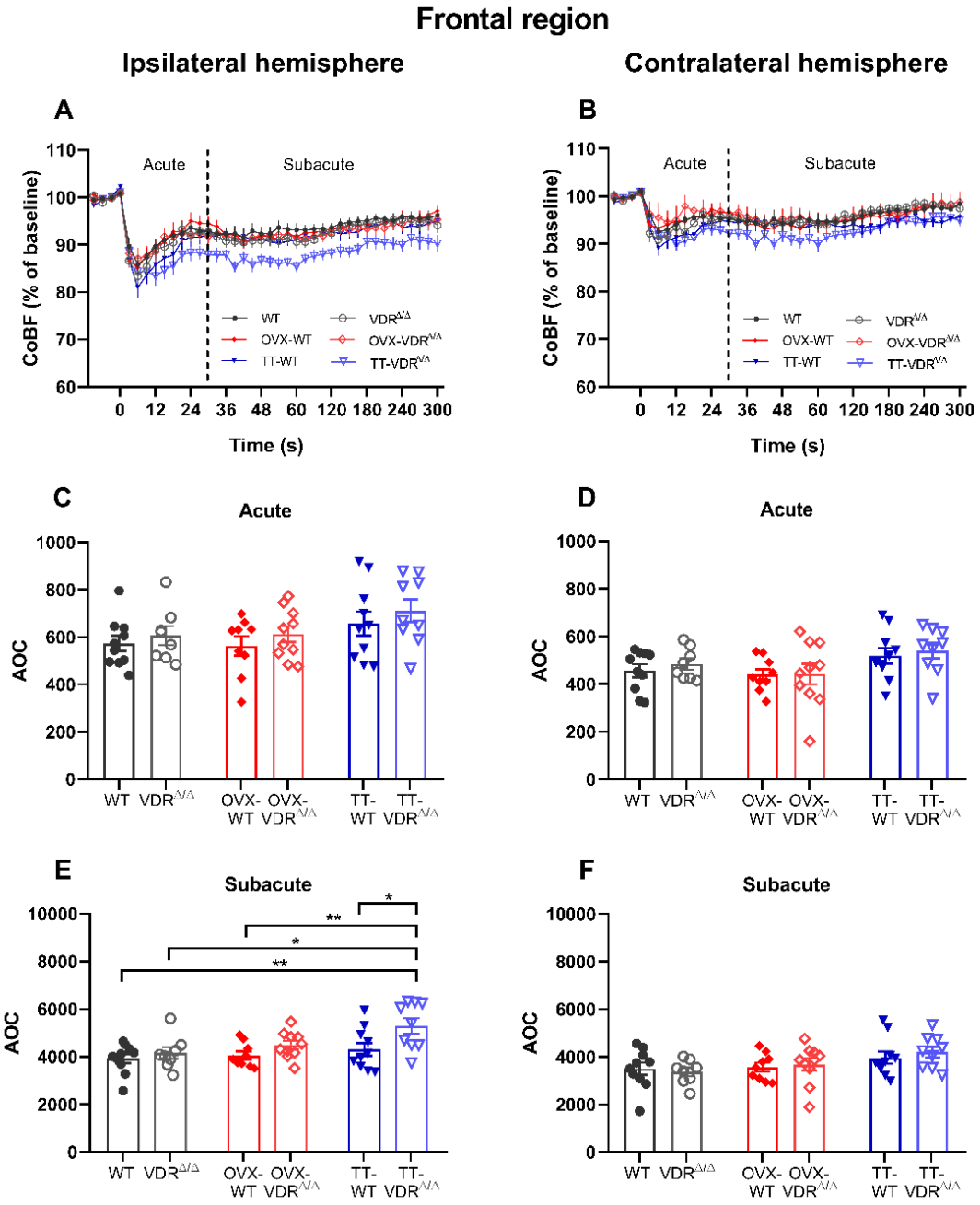

Figure 10. Cerebrocortical blood flow (CoBF) changes after unilateral carotid artery occlusion (CAO) in the frontal cortex of intact, ovariectomized (OVX) and testosterone-treated (TT) vitamin D receptor-mutant ($VDR^{\Delta/\Delta}$) and wild-type (WT) female mice. CoBF changes were recorded in the ipsilateral (**A**) and contralateral (**B**) hemispheres. The zero point on the time scale indicates the moment of CAO, and the dashed line splits the acute (0-30 s) and subacute (31-300 s) phases of the adaptation (**A, B**). CoBF reductions were quantified as the area over the curves (AOC) for each mouse and analyzed separately in the acute (**C, D**) and subacute (**E, F**) phases of adaptation. In the acute phase, no differences were observed among the experimental groups in the two hemispheres (**C, D**). However,

in the subacute phase, TT-VDR$^{\Delta/\Delta}$ mice had significantly higher AOC values than intact females (VDR$^{\Delta/\Delta}$, WT) and OVX-WT mice, indicating a prolonged reduction in blood flow in the TT-VDR$^{\Delta/\Delta}$ group (**E**). No significant differences were found among the experimental groups in the subacute phase of the contralateral hemisphere (**F**). Data are presented as mean ± SEM, n(WT)=10, n(VDR$^{\Delta/\Delta}$)=8, n(OVX-WT)=9, n(OVX-VDR$^{\Delta/\Delta}$)=10, n(TT-WT)=10, n(TT-VDR$^{\Delta/\Delta}$)=9, two-way ANOVA followed by Tukey's post hoc test (*$p<0.05$, **$p<0.01$).

The parietal region is primarily supplied by the AACA, but additional blood supply can derive from the posterior cerebral artery (150). Figures 11A (ipsilateral hemisphere) and 11B (contralateral hemisphere) show the changes in CoBF after CAO. Based on the AOC values calculated for the acute phase, no differences were discovered among the groups (Figure 11C, D). However, in the subacute phase, TT-VDR$^{\Delta/\Delta}$ mice had higher AOC values on the ipsilateral hemisphere than WT and VDR$^{\Delta/\Delta}$ mice (Figure 11E), indicating a delayed recovery. No significant differences were found in the subacute phase on the contralateral side (Figure 11F).

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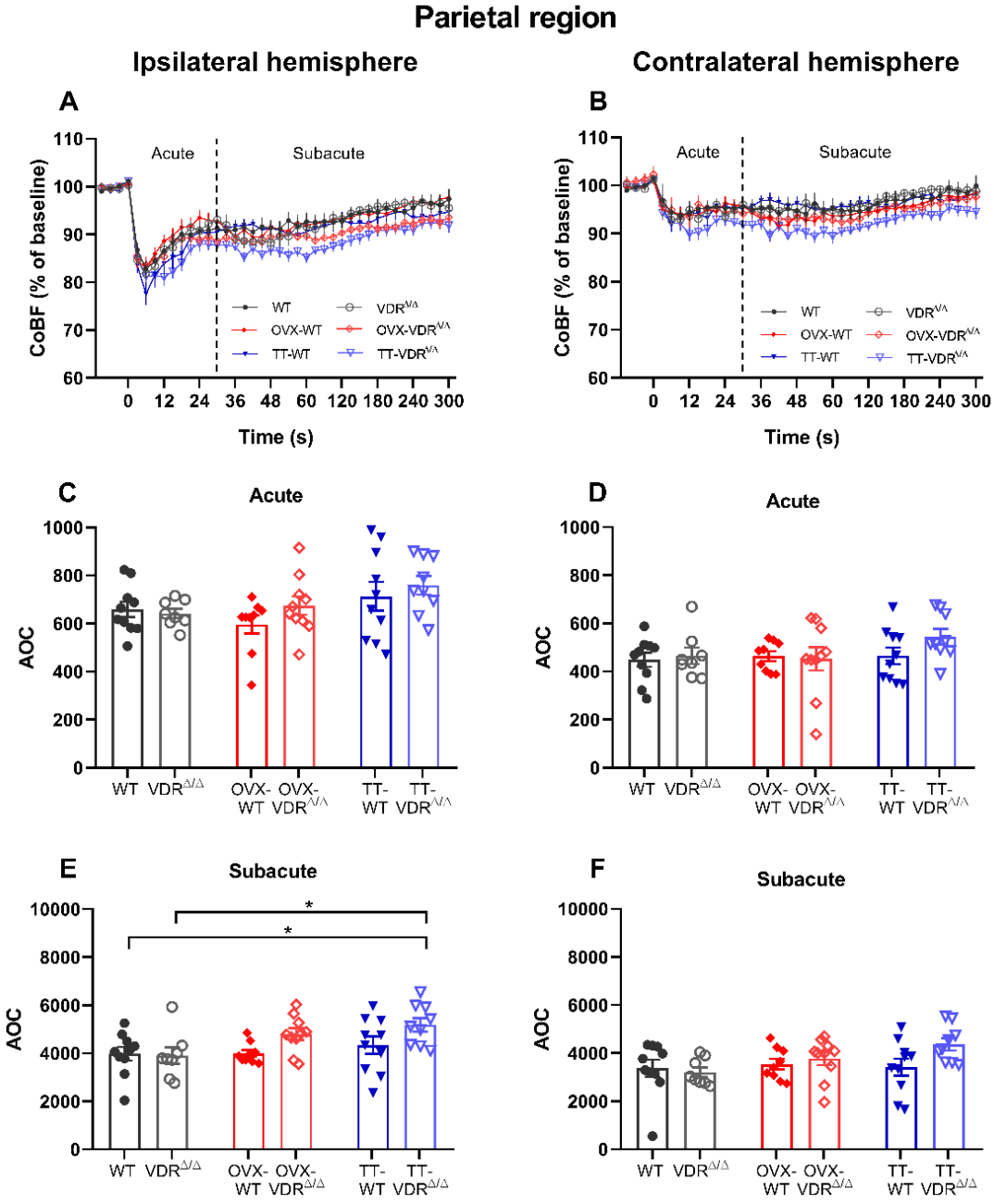

Figure 11. Cerebrocortical blood flow (CoBF) changes after unilateral carotid artery occlusion (CAO) in the parietal cortex of ovariectomized (OVX) and testosterone-treated (TT) vitamin D receptor-mutant (VDR$^{\Delta/\Delta}$) and wild-type (WT) female mice. CoBF changes were recorded in the ipsilateral (**A**) and contralateral (**B**) hemispheres. The zero point on the time scale indicates the moment of CAO, and the dashed line splits the acute (0-30 s) and subacute (31-300 s) phases of the adaptation (**A, B**). CoBF reductions were quantified as the area over the curves (AOC) for each mouse and analyzed separately in the acute (**C, D**) and subacute (**E, F**) phases of adaptation. In the acute phase, no differences were found among the experimental groups in the two hemispheres (**C, D**). However, the higher

AOC values of TT-VDR$^{\Delta/\Delta}$ mice in the ipsilateral hemisphere imply delayed recovery during the subacute phase compared to intact females (WT, VDR$^{\Delta/\Delta}$) (**E**). No significant differences were observed between the experimental groups in the subacute phase of the contralateral hemisphere (**F**). Data are presented as mean ± SEM, n(WT)=10, n(VDR$^{\Delta/\Delta}$)=8, n(OVX-WT)=9, n(OVX-VDR$^{\Delta/\Delta}$)=10, n(TT-WT)=10, n(TT-VDR$^{\Delta/\Delta}$)=9, two-way ANOVA followed by Tukey's post hoc test (*$p<0.05$, **$p<0.01$).

The temporal region is mainly supplied by the MCA, which receives a significant amount of blood only from the internal carotid artery (150). Therefore, the most pronounced reduction in blood flow after CAO was observed in this ipsilateral region (Figure 12A). On the contrary, this region of the contralateral hemisphere showed the slightest decrease in CoBF after CAO (Figure 12B), as it is located the furthest from the occluded vessel. Figures 12A and 12B show the recovery patterns of CoBF in the temporal regions of both hemispheres. No significant differences were observed between the groups in the acute phases (Figure 12C, D), similar to the other regions. However, higher AOC values in the subacute phase in the ipsilateral hemisphere of the TT-VDR$^{\Delta/\Delta}$ group compared to the WT and OVX-WT groups indicate impaired adaptation capacity in TT-VDR$^{\Delta/\Delta}$ mice (Figure 12E). No significant differences were found in the contralateral hemisphere in the subacute phase (Figure 12F).

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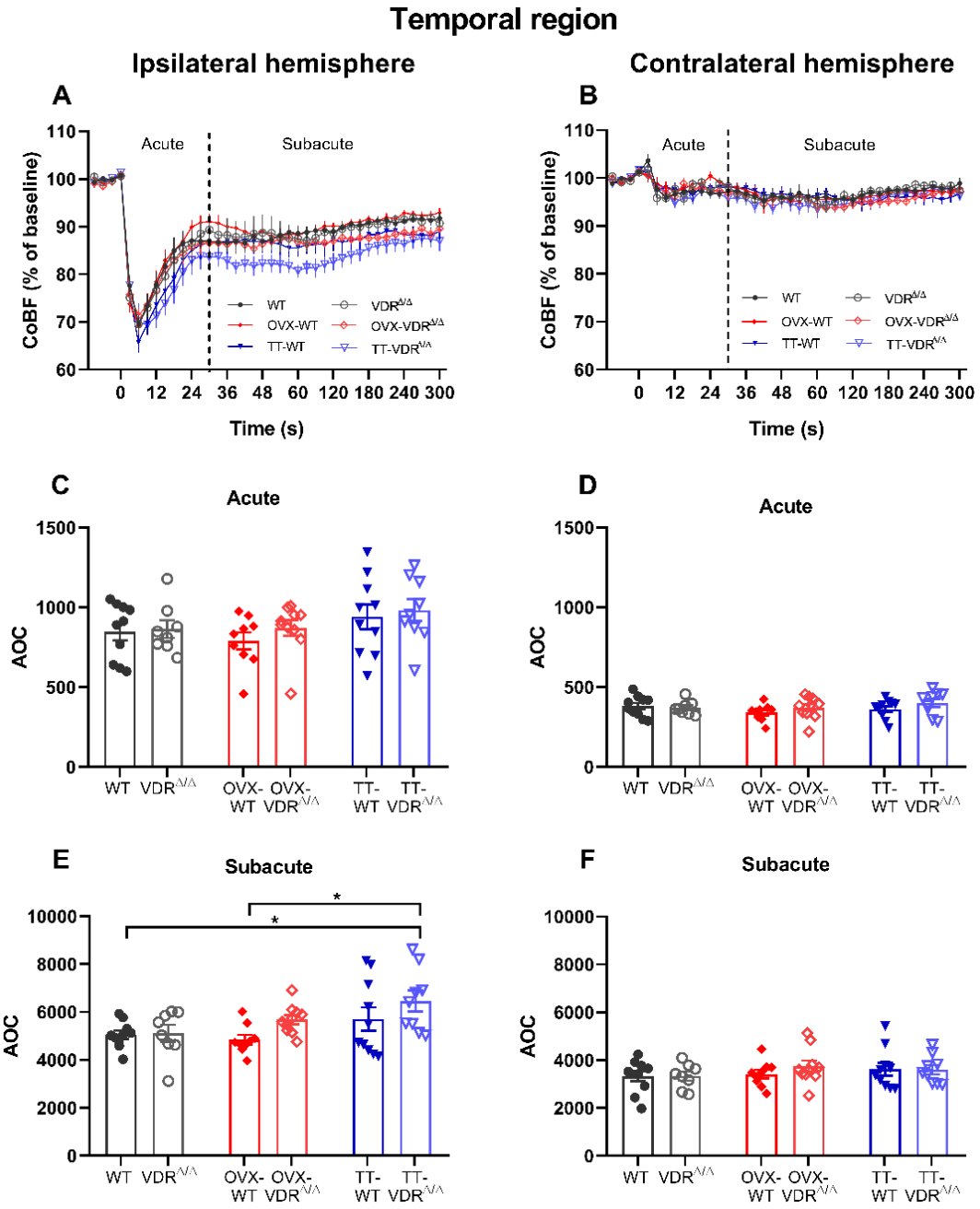

Figure 12. Cerebrocortical blood flow (CoBF) changes after unilateral carotid artery occlusion (CAO) in the temporal cortex of intact, ovariectomized (OVX) and testosterone-treated (TT) vitamin D receptor-mutant (VDR$^{\Delta/\Delta}$) and wild-type (WT) female mice. CoBF changes were recorded in the ipsilateral (**A**) and contralateral (**B**) hemispheres. The zero point on the time scale indicates the moment of CAO, and the dashed line splits the acute (0-30 s) and subacute (31-300 s) phases of the adaptation (**A, B**). CoBF reductions were quantified as the area over the curves (AOC) for each mouse and analyzed separately in the acute (**C, D**) and subacute (**E, F**) phases of adaptation. In the acute phase, no differences were observed among the experimental groups in the two hemispheres (**C, D**). However,

in the ipsilateral hemisphere, the TT-VDR$^{\Delta/\Delta}$ group showed impaired compensation during the subacute phase compared to the WT and OVX-WT groups, which can be identified by the significantly higher AOC values (**E**). No significant differences were found between the experimental groups in the subacute phase of the contralateral hemisphere (**F**). Data are presented as mean ± SEM, n(WT)=10, n(VDR$^{\Delta/\Delta}$)=8, n(OVX-WT)=9, n(OVX-VDR$^{\Delta/\Delta}$)=10, n(TT-WT)=10, n(TT-VDR$^{\Delta/\Delta}$)=9, two-way ANOVA followed by Tukey's post hoc test (*$p<0.05$, **$p<0.01$).

5. Discussion

Vitamin D has been implicated in the regulation of several biological processes beyond calcium and bone homeostasis, including cardiovascular function (27). Additionally, VDD - representing a global health issue - has been associated with chronic diseases such as cancer, cognitive impairment, autoimmune-, neurological-, and cardiovascular disorders (4). Although growing evidence suggests the link between VDD and cerebrovascular pathologies (2, 3), the impact of VDD on cerebrovascular circulation is not yet fully understood. Furthermore, women and men exhibit different prevalence of cerebrovascular disorders, which may be related to a possible interaction between vitamin D and sex steroids (113). Postmenopausal women and women in hyperandrogenic states (e.g. PCOS) are also considered risk groups for cardiovascular disorders, and their health condition is often accompanied by VDD (123, 136, 152). Therefore, understanding the interaction between vitamin D signaling and sex steroids in terms of cerebrovascular disorders is of utmost importance. In our experiments, we examined the impact of disrupted VDR signaling on cerebral circulation in intact, ovariectomized, and testosterone-treated female mice, as well as in male mice (72).

5.1. Gender differences in the effects of disrupted vitamin D signaling on the cerebral circulation

5.1.1. The impact of disrupted vitamin D signaling on the cerebral circulation in males

Vitamin D appears to modulate endothelial function, inflammatory and immune responses, oxidative stress, cell proliferation, and matrix homeostasis; therefore, it seems to largely contribute to maintaining cardiovascular health (7). Consequently, great attention has been paid to VDD in experimental and clinical vascular research to develop effective preventive strategies against cardio- and cerebrovascular pathologies associated with VDD. Various methods are used to induce VDD and investigate its consequences in animal models. In rodents, the effects of VDD are mainly investigated by applying a vitamin D-deficient chow or employing genetically modified animals (153, 154). The development of VDD requires the consumption of vitamin D-deficient chow for at least 6-8 weeks, and its success is usually confirmed by measuring serum 25-hydroxyvitamin D concentration (63, 155, 156). Different methods of genetic manipulation are also employed to model VDD. For instance, targeted ablation of 1-α-hydroxylase enzyme,

which is responsible for the second hydroxylation of vitamin D during its metabolism in the kidneys, is used to hinder the activation of vitamin D (153, 157). Moreover, gene-targeted ablation of VDR is a frequently used method to investigate the consequences of the absence of VDR signaling, in which case the beneficial biological effects of vitamin D are lost since the embryogenic state (23, 158). One version is when the full VDR gene is disrupted, resulting in the total loss of VDR expression (159, 160). In other models, including ours, VDR is still expressed but lacks a zinc finger, which is necessary for DNA binding, causing functional inactivity (23, 50, 158). In the latter model, even if vitamin D is present, its genomic effects are lost (23). However, the non-genomic effects of vitamin D may not be fully blocked. The literature is controversial about whether the non-genomic actions of vitamin D require functioning nuclear VDRs (20-22). Erben et al. also investigated whether the non-genomic effects of vitamin D mediated by membrane receptors are present in mice with functionally inactive nuclear VDRs (23). It has been reported that high doses of vitamin D metabolites cause rapid intestinal calcium absorption and hyperproliferation in keratinocytes in the skin *via* membrane receptors of vitamin D (23). However, when Erben et al. treated the mutant mice with vitamin D metabolites, they did not observe any change in calcium homeostasis or keratinocyte proliferation in mutant mice, unlike in control mice (23). They also showed that the rapid increase in intracellular calcium in response to vitamin D was not present in osteoblast cultures isolated from the mutant mice, suggesting that the membrane receptor-mediated activity of vitamin D has limited importance in these mutant mice (23). Therefore, we assume that the actions mediated by membrane receptors in our mice carrying functionally inactive VDRs have negligible effects. However, understanding the role of non-genomic effects of vitamin D in the cerebrovascular system requires further studies. Our mouse model mimics the rare human hereditary disorder known as vitamin D-dependent rickets type II, which is characterized by the unresponsiveness of VDR to vitamin D (158, 161). The clinical features of this disorder include typical symptoms of VDD, such as hypocalcemia, rickets, growth retardation, bone pain, and muscle weakness (161). Interestingly, these patients suffer from alopecia (161, 162), which is also characteristic of VDR-mutant mice (158, 163, 164). However, this feature is likely not attributed to the absence of vitamin D but to a defect in hair follicular homeostasis due to ligand-independent actions of VDR (163-165).

Employing the above-mentioned experimental mouse model, we recently discovered severe functional consequences of ablated vitamin D signaling in cerebrocortical adaptation to unilateral CAO in male mice (72). Unilateral CAO is a well-established experimental technique that mimics the pathophysiological conditions following the complete occlusion of a major artery supplying the brain, allowing the investigation of the efficiency of the compensatory mechanisms for blood loss (150). By using this technique, we previously found that the recovery after CAO was significantly slower in VDR-mutant male mice compared to the controls since they suffered more severe and prolonged hypoperfusion in certain cerebrocortical regions (temporal and parietal), which indicated impaired vasoregulation and blood flow redistribution after CAO (72). Importantly, we also reported morphological alterations of leptomeningeal collaterals in male mice with functionally inactive VDRs (72). Leptomeningeal collaterals can deliver blood to unsupplied territories from other areas; therefore, their reduction in number and deformation (increased tortuosity, shifted anastomotic line) may result in less efficient compensation after CAO (72, 166). In our current experiments, first, we aimed to reveal additional mechanisms underlying the impaired adaptation of VDR-mutant male mice. Therefore, we examined the impact of VDR inactivity on the compensatory blood flow increase in the contralateral carotid artery after unilateral CAO since the extracranial collateral circulation may also exert a considerable influence on the efficiency of the cerebrocortical adaptation to CAO by providing an alternative route for the blood flow (167). Following CAO, the blood flow is disrupted by way of the internal carotid artery and the MCA, which is the primary supplier of the temporal region (150). The blood flow sharply decreases in the ipsilateral hemisphere after CAO, but it shortly starts to return to the baseline level due to the activation of compensatory mechanisms (150). As an initial defense, the blood is redistributed in the large arteries of the circle of Willis (166, 167), and the AACA - supplying the frontal and parietal regions in both hemispheres - can provide additional supply from the other hemisphere to the hypoperfused area (150, 151). Due to the presence of an intact carotid artery, negligible blood flow disturbance is expected in the contralateral hemisphere after CAO. Therefore, increased blood flow in the contralateral internal carotid artery may enhance compensation following CAO through effective redistribution (74, 168). However, we found no significant differences in the increase in blood flow or vascular conductance of the intact carotid artery among

the male experimental groups ($VDR^{\Delta/\Delta}$♂, WT♂). This finding indicates that the impaired cerebrocortical adaptation in male VDR-mutant mice is not due to altered reactivity of the intact carotid artery to CAO. Nevertheless, the vasoregulatory dysfunction and the compromised collateral morphology that developed due to the absence of VDR signaling can be responsible for the prolonged CoBF reduction in male mice (72).

5.1.2. The impact of disrupted vitamin D signaling on the cerebral circulation in healthy female mice

Although the adverse functional consequences of ablated VDR signaling on the cerebrocortical microcirculation were previously confirmed in male mice (72), it is still unclear whether sex steroids influence the acute compensatory mechanisms after CAO. Therefore, we aimed to analyze the recovery patterns of CoBF after CAO in three different cerebrocortical regions (frontal, parietal, temporal) in VDR-deficient females. VDD is associated with impaired vascular reactivity, inflammation, and endothelial dysfunction, which can adversely affect cerebrovascular function (3, 72). VDD reduces the bioavailability of NO, the essential mediator of endothelial-dependent vasodilation (15). Moreover, VDD is associated with higher levels of ROS and inflammatory mediators, ultimately leading to endothelial dysfunction and enhanced vasoconstriction due to an altered balance of prostanoid mediators (3, 15). Thus, we assumed that VDD may worsen the efficiency of the cerebrovascular adaptation to a large artery occlusion. Surprisingly, in intact female mice, VDR deficiency by itself did not impair the adaptation of the cerebrocortical circulation to CAO since no differences were observed in CoBF between the intact $VDR^{\Delta/\Delta}$ and WT females in any of the investigated regions. This well-preserved compensatory function in females strongly implies sex dimorphism in the effect of ablated vitamin D signaling, as previously, more dramatic alterations were discovered in males (72). In accordance with these findings, Pál et al. reported that VDD caused altered vascular reactivity, inward hypertrophic remodeling, and endothelial dysfunction in anterior cerebral arteries of male rats (141, 142). Interestingly, these alterations were not present in vitamin-D-deficient female rats (142), which also suggests gender differences in the cerebrovascular manifestation of VDD.

Next, we aimed to investigate the two compensatory pathways that might be partially responsible for the efficiency of the cerebrocortical adaptation to CAO in females as well: the impact of VDR-inactivity on the extracranial collateral circulation and on the pial

collateral circulation. Thus, we examined the compensatory increase in blood flow in the contralateral carotid artery (i.e., the extracranial collateral circulation) that may improve the blood flow redistribution after CAO in intact female mice. We found no significant differences either in the increase in the blood flow or the vascular conductance of the intact carotid artery between the female experimental groups ($VDR^{\Delta/\Delta}$, WT). This finding indicates that VDR inactivity does not impair the increase in the contralateral carotid artery blood flow in females, which may promote effective blood flow redistribution after CAO. Interestingly, Sipos et al. reported that carotid arteries isolated from rats exhibit gender and vitamin D-dependent functional alterations, with more unfavorable effects in males (91). However, we found that VDR-inactivity did not impair carotid artery reactivity *in vivo*, regardless of gender.

In addition to the extracranial collateral circulation, leptomeningeal collaterals between the branches of the ACA and MCA can significantly impact the outcome of a large artery blockade by mediating the blood flow to an area with higher demands (151, 166). Accordingly, they can improve the perfusion of the temporal region at the expense of the frontal-parietal regions after CAO (150). Therefore, to evaluate the ability of MCA-to-ACA collaterals to alleviate hypoperfusion of the temporal region, their morphology was evaluated in intact females (72). Our results showed that only the number of collaterals decreased in $VDR^{\Delta/\Delta}$ females compared to the WT group, while the tortuosity and the localization of the vessels did not change. In contrast with this finding, in males, in addition to the reduced number of collaterals, both the increased tortuosity and the shifted anastomotic line indicated more severe alterations caused by VDR inactivity (72). This alteration may partly explain the less efficient cerebrocortical adaptation to CAO in males compared to females, as the collateral vascular network can significantly influence the outcome of a large artery occlusion in the brain (169). For instance, the infarct size after MCA occlusion is inversely associated with the number and diameter of collaterals, whereas it is directly related to the area of the MCA (170). The measured distance of the anastomotic line from the midline represents the border of brain areas supplied by the ACA and MCA; therefore, a shift in localization would indicate a change in the ratio of the ACA and MCA areas (72, 166). Besides, increased vascular tortuosity is associated with disturbed hemodynamic conditions (171), which may result in abnormal shear stress on the endothelium and, consequently, impair flow-induced dilatation (72, 172). Vitamin

D regulates genes involved in cell proliferation, differentiation, and migration, while sex steroids can also exert an effect on these processes (2, 3, 107, 109). Moreover, VEGF is a key mediator of angiogenesis (48, 173, 174), and VEGF receptor expression is also increased by vitamin D (46, 47). A rodent study reported that the number of collaterals is not only dependent on sex (126), which suggests that VDR deficiency may be a responsible factor for the reduction in collateral number, independently of gender. Nevertheless, the gender differences in tortuosity and localization of collaterals still imply a sex-dependent influence on the consequences of VDR inactivity. Tortuosity was reported to increase progressively with time after the formation of collaterals due to proliferation, even in three-month-old mice compared to their post-natal state (171). During collateral development (which occurs during embryogenesis and the first postnatal weeks), sex hormone levels are low in mice and start to rise only after the end of collaterogenesis (175). Therefore, it is more likely that gender differences developed during adulthood when sex steroids may influence the consequences of VDD. However, the exact underlying mechanisms have yet to be revealed.

Cardiovascular parameters that significantly influence cerebral autoregulation, such as blood pressure and blood gas tension, were also measured. The impact of VDD on blood pressure was examined more thoroughly in male than in female mice; however, the results are inconsistent. Some studies reported elevated blood pressure in VDR-deficient mice that may be attributed to excessive renin expression (50, 176, 177), but others observed no differences (72, 160, 178), or even lower systolic values (178). This discrepancy may be due to methodological differences, such as the age of animals, their diet (i.e., conventional chow or rescue diet), or measurement technique (e.g. anesthesia). In our experiments, male VDR-mutant mice did not develop higher blood pressure compared to control mice. Similarly, no significant differences were found in blood pressure between intact $VDR^{\Delta/\Delta}$ and WT females. Moreover, even the arterial blood gas tensions (i.e., pCO_2, pO_2) did not differ between our female groups; therefore, we could rule out the possibility that these factors contributed to CoBF alterations after CAO.

5.2. Combined effects of disrupted vitamin D signaling and estrogen deficiency on the cerebrocortical adaptation in female mice

Considering the less adverse consequences of disrupted vitamin D signaling on the cerebrocortical adaptation to CAO in females, we hypothesized that higher levels of

estrogen might provide a level of protection against the harmful effects of VDR inactivity. Importantly, postmenopausal women show a higher prevalence of VDD and are more susceptible to developing cardiovascular disorders (114, 179). Estrogen enhances NO-dependent vasodilation by increasing its production by stimulating endothelial NO synthase expression and activity (82). Moreover, estrogen shifts prostanoid production towards vasodilator prostanoids by increasing prostacyclin levels and consequently improving the relaxation of the vessels (84). Vitamin D increases the bioavailability of NO, reduces the levels of proinflammatory cytokines and oxidative stress, regulates prostaglandin synthesis, and thus prevents the development of endothelial dysfunction (3, 15). Besides, vitamin D and estrogen were reported to upregulate the expression of each other's receptors (144, 145, 180), implying a possible interaction between them. Considering the vasoprotective role of estrogen (82), it is likely that estrogen protects from the deleterious effects of VDD in females, which may explain the unaltered adaptational capacity observed in VDR-mutant females. Previous studies also suggested that ovariectomy compromises the cerebrovascular circulation (88, 134, 181). For instance, cerebral arteries of ovariectomized mice showed increased vasoconstriction with less NO-mediated dilation, but estrogen replacement reversed this effect (181). Additionally, constriction mediated by vasoconstrictor prostanoid mediators was predominant in the cerebral arteries of ovariectomized rats, while estrogen replacement made vasodilator prostanoids preeminent (88). Ovariectomized females also had larger infarct volumes, indicating the neuroprotective effect of estrogen (134). Therefore, we assumed that the estrogen-dependent vasoprotective effects may decrease after ovariectomy. For our experiments, estrogen deficiency, which is characteristic of postmenopausal women, was generated by surgical ovariectomy in VDR-deficient mice. The cerebrocortical blood flow changes were investigated five weeks after the surgery, by the time the estrus cycle of ovariectomized mice had ceased as expected (147, 182), which verified the success of the removal of ovaries. The body mass of ovariectomized females significantly increased, similar to previous findings (183, 184). Although the reason for weight gain is not entirely understood, this phenomenon might be attributed to higher food intake and the regulation of fat accumulation by estrogen (e.g. by affecting energy expenditure and protein synthesis) (183, 184). Additionally, estrogen treatment was reported to reverse this effect of ovariectomy by inhibiting food intake and abdominal

fat accumulation, suggesting a role for estrogen in the prevention of obesity (184). The effects of VDD and estrogen insufficiency on cardiovascular parameters such as blood pressure have not yet been extensively investigated in rodent models, and the results are controversial. For instance, ovariectomy was previously reported to increase blood pressure in female mice, especially when combined with VDD (183). In contrast, in our experiments, neither ovariectomy nor VDR inactivity resulted in appreciable blood pressure differences. However, it is important to note that our mice were anesthetized during the experiments, which may have altered their blood pressure compared to the normal resting values. Surprisingly, ovariectomy did not undermine the compensatory mechanisms after CAO in our experiments since no differences were observed in the adaptational capacity between the intact ($VDR^{\Delta/\Delta}$, WT) and the ovariectomized mice (OVX-$VDR^{\Delta/\Delta}$, OVX-WT). However, it is important to consider that the mice involved in the experiments were young and only experienced five weeks of estrogen deprivation. Additionally, estrogens may improve the chronic outcomes of ischemia instead of the acute blood flow changes after occlusion.

5.3. Synergistic effects of disrupted vitamin D signaling and androgen excess on the cerebrocortical adaptation in female mice

Since ovariectomy did not undermine the effect of the loss of vitamin D signaling on the adaptation of the cerebrocortical microcirculation to CAO, we hypothesized that androgen excess in females may aggravate the impact of VDR inactivity. The interaction between VDR and AR has been suggested in prostate cells, and in chondrocytes *via* AR coregulators (185, 186). Moreover, VDD was reported to alter the expression of AR proteins in male cerebral arteries but not in females, suggesting a complex interplay between androgen and vitamin D signaling (142). Interestingly, hyperandrogenism caused alterations in the vascular reactivity of thoracic aorta and skeletal muscle arterioles in rat PCOS models, but vitamin D treatment mitigated these effects (187-189). However, further studies are required to elucidate the exact crosstalk between vitamin D and androgen signaling in the cerebral circulation. Additionally, how androgens affect cerebrovascular function is well-described in males, but it is yet to be revealed in females (80). Androgens enhance vasoconstrictor thromboxane A_2 production, suppress endothelium-derived hyperpolarizing factors, and stimulate inflammatory responses, thereby generating increased vascular tone (84). In addition, androgen excess can induce

vessel wall remodeling in large arteries and thus impact vascular reactivity (141). Interestingly, vitamin-D-deficient female rats developed inward remodeling and altered reactivity with enhanced vessel tone in anterior cerebral arteries, however, only when they simultaneously suffered from hyperandrogenism and VDD, but not when VDD alone was present (142, 143). Therefore, androgen excess may be an aggravating factor in females in the cerebrovascular consequences of VDD.

To induce androgen excess in our female mice, we administered daily testosterone treatment for five weeks. Even though the serum testosterone concentrations of our control mice ($VDR^{\Delta/\Delta}$, WT) were below the detection limit, based on the available literature data (190), the testosterone concentrations measured in our treated groups were several times higher than normal concentrations in female mice. This proved the efficiency of the five-week-long transdermal treatment that increased the testosterone concentrations to the range between the normal testosterone levels reported in female and male mice (23, 190), which corresponds to the increase observed in hyperandrogenic women (191). The body mass of testosterone-treated females was also significantly increased, consistent with previous reports (156, 192). Interestingly, while androgen treatment increases muscle mass in males, females were reported to develop higher body mass due to different mechanisms (192). For example, the mass of adipose tissue increases due to higher food intake and lower metabolic rate (192). Literature data are controversial about the effect of androgen excess on blood pressure in females. For instance, rats in a PCOS model were reported to develop high blood pressure (193). In contrast, another study indicated that neither testosterone nor VDD influenced the blood pressure of anesthetized rats (142). Consistent with this finding, in our study, testosterone-treated females did not develop higher blood pressure than intact females, which confirms that the adaptive capacity of testosterone-treated mice could not be altered by changes in blood pressure. Interestingly, the cerebrocortical adaptation of TT-$VDR^{\Delta/\Delta}$ mice was most impaired compared to the other experimental groups, as they suffered prolonged hypoperfusion in all ipsilateral regions (i.e., temporal, frontal, parietal) during the subacute phase of adaptation to CAO. The temporal region of TT-$VDR^{\Delta/\Delta}$ mice showed the slowest recovery, while the frontal and parietal regions were also affected. The reason behind this phenomenon may be the draining effect through the leptomeningeal collaterals, as they try to supply the more ischemic temporal area at the

expense of the frontal-parietal regions (150). It was also reported that both the pial collaterals and the large vessels of the circle of Willis must dilate to improve the perfusion (150). Since both VDD and the excess of androgens compromise endothelial function and vascular reactivity, when these two factors are combined, they may be strong enough to impair the compensatory mechanisms to the extent that did not appear in intact $VDR^{\Delta/\Delta}$, ovariectomized females (i.e., OVX-$VDR^{\Delta/\Delta}$, OVX-WT) and testosterone-treated wild-type (TT-WT) mice. Therefore, our results imply that the deleterious cerebrovascular impact of VDD may only manifest in young females when VDD is combined with hyperandrogenism.

When examining the pial collateral circulation, we found gender differences in the impact of VDR inactivity on the morphology of collaterals. While not only vitamin D but also sex steroids may influence angiogenesis (46, 108, 194), it remains in question whether estrogen deficiency or androgen excess alters the density, tortuosity, or localization of these vessels. Unfortunately, the current method for investigating the morphology of leptomeningeal collaterals is limited because the parameters cannot be examined in the same mouse before and after the five weeks of hormone treatment. However, since angiogenesis occurs only under pathological conditions in the brain, such as chronic ischemia or tumor growth (195), and collateral rarefaction develops during months of aging (196), it is hardly likely that the collaterals would undergo considerable morphological alterations (i.e., changes in number, localization or tortuosity) in young, adult mice during the five weeks of hormone imbalance. Therefore, we assume that the short-term estrogen deficiency or androgen excess would not cause any severe alterations in the collateral morphology without stimulating angiogenesis (e.g. chronic ischemia) (195). Nevertheless, as wall morphology and function of the large arteries of the circle of Willis may still be altered by concomitant VDD and hyperandrogenism (142), this could potentially influence the efficiency of blood redistribution after occlusion. Specifically, reduced endothelium-mediated dilation due to decreased eNOS expression and enhanced vascular tone due to excess production of vasoconstrictor prostanoid may be responsible for the diminished adaptive capacity of TT-$VDR^{\Delta/\Delta}$ mice by compromising the acute vasodilatory responses to occlusion. To sum up our findings, Table 6 summarizes the combined effects of disrupted vitamin D signaling and hormonal status on different aspects of the cerebral circulation (i.e., cerebrocortical adaptation to CAO, blood flow

elevation in the intact carotid artery after CAO, morphology of leptomeningeal collaterals) which were examined or previously reported (72).

Table 6. Combined effects of disrupted vitamin D signaling and hormonal status on the cerebral circulation. Disrupted vitamin D signaling did not affect the cerebrocortical adaptation to unilateral carotid artery occlusion (CAO), and the blood flow increase in the contralateral carotid artery in healthy female mice, but slightly impacted the morphology of pial collaterals indicated by their reduced number. On the contrary, Pál et al. reported diminished cerebrovascular adaptation to CAO in male mice – due to the more seriously altered morphology of collaterals (72), but not because of an impaired compensatory blood flow increase in the contralateral carotid artery – indicating gender differences in the effect of disrupted vitamin D signaling. The reason behind the gender differences might be the interaction between sex steroids and vitamin D, as in females, the simultaneous presence of disrupted vitamin D signaling and androgen excess undermined the cerebrocortical adaptation to CAO, similar to our findings in males. Surprisingly, estrogen deficiency did not aggravate the impact of disrupted vitamin D signaling on the cerebrocortical adaptation. In the table below, the "✓" mark indicates well-preserved function, while the "✓" mark refers to slight alteration, and × indicates impairment developed in vitamin D receptor deficiency. The results obtained from the cerebrocortical blood flow measurements and the morphological analysis of males were not included in this thesis (72).

	♀			♂
	Disrupted vitamin D signaling			Disrupted vitamin D signaling
	Intact	Estrogen deficiency	Androgen excess	
Cerebrocortical adaptation to CAO	✓	✓	×	× (72)
Contralateral blood flow elevation	✓			✓
Morphology of pial collaterals	✓			× (72)

Endocrine disorders characterized by excess androgens can occur at any stage of a woman's life, and they are not only associated with an increased risk of cardiovascular disorders but are also often accompanied by VDD (135, 197). Women with androgen excess, particularly PCOS, develop symptoms (e.g. endothelial dysfunction, arterial stiffness, chronic inflammation, hypertension) that largely contribute to their higher risk of ischemic stroke (123). In addition, VDD was associated with exacerbated symptoms

of PCOS, which could be adversely improved by high-dose vitamin D supplementation according to randomized controlled trials (139, 140). The present study shows the functional impairment of the cerebrovascular circulation that developed due to the simultaneous presence of disrupted vitamin D signaling and hyperandrogenism, which implies that this health condition could even directly worsen the outcome of an obstructive vascular disease in the brain, like ischemic stroke. Additionally, our results highlight the need for careful management of cardiovascular disorders associated with hyperandrogenic disorders and VDD.

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6. Conclusions

In our experiments, we aimed to investigate the impact of disrupted vitamin D signaling and its interaction with altered sex hormone status on cerebrovascular adaptation to unilateral carotid artery occlusion. Our findings indicate that:

- In male mice, the compensatory increase in blood flow in the contralateral carotid artery after CAO was not compromised by VDR inactivity. Therefore, this mechanism did not contribute to the decreased cerebrovascular adaptative capacity of VDR-mutant males.

- Functional inactivation of VDR itself did not impair the cerebrovascular adaptation to CAO in intact females, to which may have contributed
  - the less severe morphological alterations of leptomeningeal collaterals,
  - and the preserved reactivity of the intact contralateral carotid artery, which might provide sufficient compensatory blood flow increase after CAO.

- Estrogen deficiency did not aggravate the effect of disrupted VDR signaling on the cerebrovascular adaptation to CAO, as no differences were found in the recovery of cerebrocortical blood flow after CAO between intact and ovariectomized female mice.

- VDR-inactivity combined with hyperandrogenism impaired the acute blood flow compensation following CAO in female mice since the simultaneous presence of androgen excess and disrupted vitamin D signaling resulted in prolonged hypoperfusion in all regions of the ipsilateral cortex. These findings imply a vasoregulatory dysfunction that may directly aggravate the outcome of cerebrovascular diseases (e.g. ischemic stroke) when vitamin D deficiency and hyperandrogenism coexist.

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7. Summary

Vitamin D deficiency represents an emerging cerebrovascular risk factor as it has been associated with the pathogenesis of cerebrovascular disorders, including ischemic stroke. Our previous findings showed the detrimental effects of vitamin D receptor (VDR) inactivity on the cerebrovascular function in male mice, characterized by more severe and prolonged blood flow reduction in the cerebral cortex after unilateral carotid artery occlusion (CAO) due to – at least partly – the altered morphology of leptomeningeal collaterals. To reveal if impaired extracranial collateral circulation also underlies the less efficient adaptation of VDR-mutant male mice, we examined the compensatory blood flow increase in the contralateral carotid artery, but we did not find any alterations in it. Nevertheless, the prevalence of cerebrovascular diseases might also be influenced by sex hormonal status, indicated by the heightened vulnerability of men, postmenopausal, and hyperandrogenic women compared to young, healthy women. Therefore, we aimed to investigate the interaction between vitamin D signaling and sex steroids in terms of cerebrovascular disorders by examining the impact of disrupted VDR signaling on the cerebrocortical microcirculation in intact, ovariectomized, and testosterone-treated female mice. Interestingly, unlike in males, disrupted vitamin D signaling by itself did not compromise the cerebrovascular adaptation to CAO in intact females, which might be attributed to the well-preserved leptomeningeal and extracranial collateral circulation. These findings strongly imply sex dimorphism in the effect of VDR inactivity on cerebrovascular function. Next, we investigated whether estrogen deficiency or hyperandrogenism aggravates the effect of VDR inactivity in females. Surprisingly, while ovariectomy did not undermine compensatory mechanisms following CAO, androgen excess combined with VDR inactivity resulted in prolonged hypoperfusion in the ipsilateral cerebral cortex. These findings suggest that the cerebrovascular consequences of disrupted VDR signaling are less pronounced in females, providing a level of protection even after ovariectomy. In contrast, even short-term androgen excess with the loss of VDR signaling may lead to unfavorable outcomes of cerebrovascular disorders, highlighting the complex interplay between androgens and vitamin D.

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9. Bibliography of the candidate's publications

Publications related to the dissertation

Nagy D, Hricisák L, Walford GP, Lékai Á, Karácsony G, Várbíró S, et al. Disruption of Vitamin D Signaling Impairs Adaptation of Cerebrocortical Microcirculation to Carotid Artery Occlusion in Hyperandrogenic Female Mice. Nutrients. 2023;15(18):3869.

IF: 5.9

Pál É, Hricisák L, Lékai Á, **Nagy D**, Fülöp Á, Erben RG, et al. Ablation of Vitamin D Signaling Compromises Cerebrovascular Adaptation to Carotid Artery Occlusion in Mice. Cells. 2020;9(6).

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