

Université de Montréal

**Warfarin-induced vitamin K deficiency is associated
with cognitive and behavioral perturbations, and
alterations in brain sphingolipids in rats**

by

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Ce mémoire intitulé :

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with cognitive and behavioral perturbations, and
alterations in brain sphingolipids in rats**

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Résumé

La Vitamine K (VK) est largement reconnue pour son rôle dans la coagulation sanguine toutefois, de plus en plus de travaux indiquent son implication dans la fonction cérébrale. La VK est requise pour l'activation de différentes protéines, par exemple la protéine Gas6, et la ménaquinone-4 (MK-4), le principal vitamère K dans le cerveau, est impliquée dans le métabolisme des sphingolipides. Dans un rapport précédent, nous avons montré qu'un régime alimentaire faible en VK tout au long de la vie était associé à des déficits cognitifs chez des rats âgés. La warfarine sodique est un puissant antagoniste de la VK qui agit en bloquant le cycle de la VK, provoquant un «déficit relatif de VK » au niveau cellulaire. À la lumière du rôle émergent de la VK dans le cerveau, la warfarine pourrait représenter un facteur de risque pour la fonction cérébrale. Ce travail est donc pertinente en raison de la forte proportion d'adultes traités à la warfarine sodique. Dans la présente étude, 14 rats mâles Wistar ont été traités avec 14 mg de warfarine/kg /jour (dans l'eau potable) et des injections sous-cutanées de VK (85 mg/kg), 3x/sem, pendant 10 semaines. Quatorze rats témoins ont été traités avec de l'eau normale et injectés avec une solution saline. Les rats ont été soumis à différents tests comportementaux après quoi les niveaux de phylloquinone, MK-4, sphingolipides (cérébroside, sulfatide, sphingomyéline, céramide et gangliosides), et les sous-types de gangliosides (GT1b, GD1a, GM1, GD1b), ont été évalués dans différentes régions du cerveau. Comparativement aux rats du groupe contrôle, les rats traités à la warfarine présentaient des latences plus longues au test de la *piscine de Morris* ($p < 0,05$) ainsi qu'une hypoactivité et un comportement exploratoire plus faible au test de « l'*open field* » ($p < 0,05$). Le traitement par warfarine a également entraîné une diminution spectaculaire du niveau de MK-4 dans toutes les régions du cerveau ($p < 0,001$), une altération des concentrations de sphingolipidiques, en particulier dans le cortex frontal et le mésencéphale ($p < 0,05$), et une perte de différences régionales sphingolipidiques, notamment pour les gangliosides. Le traitement par warfarine a été associé à un niveau inférieur de GD1a dans l'hippocampe et un niveau supérieur de GT1b dans le cortex préfrontal et le striatum. En conclusion, la déficience en VK induite par warfarine altère les niveaux de VK et

sphingolipides dans le cerveau, avec de potentiels effets néfastes sur les fonctions cérébrales.

Mots-clés : La vitamin K, MK-4, warfarin, cognition, sphingolipides, rats.

Abstract

Vitamin K (VK) is widely known for its role in blood coagulation, however many studies suggest its involvement in brain function. VK is required for the activation of various cerebral proteins (e.g., Gas6) and menaquinone-4 (MK-4), the main K vitamer in brain, is involved in sphingolipid metabolism. Furthermore, life-long intake of a low VK diet has been associated with cognitive deficits in old rats. Warfarin (W) is a potent VK antagonist that acts by blocking the VK cycle causing a “*relative VK deficiency*” at the cellular level. In light of this and the emerging role of VK in brain, W could represent a risk factor for cerebral function. The finding of this study is important according to the large proportion of adults with thromboembolic diseases being treated with warfarin drugs. This study was conducted in a rat model where the impact of W was investigated with respect to cognition, behavior, and brain menaquinone-4 (MK-4) and sphingolipid status. Fourteen Wistar male rats were treated with 15 mg W/kg/d (in drinking water) and subcutaneous VK (85 mg/kg), 3X/wk, for 10 wks; 14 control rats were treated with normal water and injected with saline. At the end of the treatment period, rats were subjected to different behavioral tests, afterwhich their brains assessed for VK (phylloquinone and MK-4) and sphingolipids (gangliosides, ceramides, cerebrosides, sphingomyelin and sulfatides) and gangliosides subtypes (GT1b, GD1a, GM1, GD1b). Mean latencies to find the hidden platform were higher in the W compared to the control group ($p < 0.05$) suggesting cognitive deficits as well as hypoactivity and lower exploratory behaviour in the open field test ($p < 0.05$). Warfarin treatment also resulted in a dramatic decrease in MK-4 concentration in all brain regions ($p < 0.001$), altered sphingolipid level, especially in frontal cortex and midbrain ($p < 0.05$), and in a loss of sphingolipid regional differences, notably for gangliosides. W treatment was associated with lower GD1a in the hippocampus and higher GT1b in the striatum and prefrontal cortex. In conclusion, warfarin-induced VK deficiency alters VK and sphingolipid status in brain with potential detrimental effects on brain functions.

Keywords : Vitamin K, MK-4, warfarin, cognition, sphingolipids, rat.

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List of abbreviations

AD	Alzheimer's Disease
A β	Amyloid β
EPM	Elevated plus maze
GGCX	γ -glutamyl carboxylase
Gla	Gamma-carboxyglutamic acid
Glu	Glutamic acid
GRP	Gla rich protein
HD	Huntington's Disease
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
KH2	Hydroquinone
KO	Vitamin K 2,3-epoxide
LDL	Low density lipoprotein
MK-4	Menquinone-4
MPG	Matrix Gla protein
MWM	Morris water maze
OF	Open field
PIVKA	Protein induced by vitamin K absence or antagonism
PRGP	Prolin-rich Gla protein
SMS	Sphingomyelin synthase
TAM	Tyro3, Axl, and Mer
TMGs	Transmembrane Gla proteins
TRGLP	Triacylglycerol-rich lipoprotein
UBIAD1	UBiA prenyltransferase domain-containing protein 1
VK	Vitamin K
VKDP	Vitamin K dependent protein
VKOR	Vitamin K oxido-reductase

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Introduction

The history of vitamin K (VK) dates back to 1929, when it was discovered for its role in blood coagulation. Specifically, it acts as a cofactor for the γ -glutamyl carboxylase (GGCX) in the posttranslational carboxylation reaction of γ -carboxyglutamic acid (Gla) from glutamic acid (Glu), an amino acid common to all VK dependent proteins (VKDP) (ie. Gas6 and protein S), and extends the role of this vitamin beyond coagulation (reviewed in Ferland 2012b).

Gas6 and protein S are ligands for the receptor tyrosine kinases of the TAM family (Tyro3, Axl, and Mer) and are involved in cell signalling actions. Specifically, protein Gas6 has been involved in cell growth and survival, myelination, chemotaxis and mitogenesis. As for protein S, it has been shown to offer neuronal protection during ischemic/hypoxic injury (through its TAM-related actions) in addition to possessing antithrombic properties (Varnum, Young et al. 1995).

Studies have shown the presence of VK in brain tissue, mostly in the form of menaquinone-4 (MK-4) (Thijssen and Driittij-Reijnders 1996). The concentration of MK-4 in the brain of 6 and 21- month old rats was reported at more than 98% of the total VK (Carrie, Portoukalian et al. 2004; Carrie, Belanger et al. 2011). In brain, MK-4 is unevenly distributed among regions; it is present in highest concentrations in midbrain and pons medulla, whereas cerebellum, olfactory bulb, thalamus, hippocampus, and striatum contain the lowest levels (Carrie, Portoukalian et al. 2004). Furthermore, MK-4 concentrations increase as a function of phylloquinone intake, decreases with age and, are higher in female than in male rats (Ronden, Thijssen et al. 1998; Huber, Davidson et al. 1999).

VK is also important to brain as its role in sphingolipid metabolism. Sphingolipids are major components of cell membranes and are highly present in the various cell types of the nervous system (Bartke and Hannun 2009). In addition to their structural roles, sphingolipids play critical roles in cellular events such as proliferation, differentiation, senescence, cell-cell interaction, and transformation (Zeidan and Hannun 2007). The specific role of VK in sphingolipid synthesis was initially discovered in bacteria in the

1970s (Lev and Milford 1972; Lev and Milford 1973), and later confirmed in rats (Sundaram and Lev 1988).

Physiologically, alterations in sphingolipids metabolism have been observed in the aging process and in neurodegenerative disorders such as Alzheimer and Parkinson diseases (Fantini and Yahi 2010; van Echten-Deckert and Walter 2012)

VK status has also been shown to affect behaviour. In a study by Cochetto *et al.*, rats rendered VK deficient through diet or following treatment with the VK antagonist, warfarin, were observed to show hypoactivity and lack of exploratory behaviour (Cocchetto, Miller et al. 1985). In a recent report, lifelong consumption of a low VK diet was linked to cognitive deficit in old rats, an impairment that was associated with an alteration in the sphingolipids profile of the rats' brain (Carrie, Belanger et al. 2011).

Hence, data published thus far point to detrimental effects of a sub-optimal VK status for brain function. This finding is important in light of the large proportion of adults being treated with warfarin drugs (Božina 2010).

In the Cochetto paper, the VK status of the animals was not assessed, hence the behavioural conclusions cannot be directly linked to VK. The present study aimed to further investigate the role of VK in cognition and behaviour in warfarin-induced VK deficiency with respect to brain's VK and sphingolipid status.

Literature Review

1. Vitamin K

VK was discovered in 1929 by Henrik Dam as part of experiments on sterol metabolism. When chickens were subjected to a fat-free diet, Dam observed that animals developed subcutaneous hemorrhages and anemia. The fat soluble anti-hemorrhagic component was found in liver extracts and several plant tissues and in 1935, it was named VK in reference of the first letter of the German word *Koagulation* (Suttie 2009).

1.1. Chemical Structure of Vitamin K

All K vitamers possess a 2-methyl-1,4-naphthoquinone ring structure called menadione or K₃; the K vitamers differ based on the side chain at the 3-position which varies in length and degree of saturation. VK includes two natural forms: 1) phylloquinone, also referred to as K₁ (2-methyl-3-phytyl-1, 4-naphthoquinone), is synthesized in plants, and 2) the menaquinones (MK-n) or K₂ (2-methyl-1, 4-naphthoquinone), a family of compounds of bacterial origin (Food and Nutrition Board 2001). One of the menaquinone, menaquinone 4 (MK-4), is not a product of bacterial synthesis but is produced from phylloquinone. Animal studies have shown that this K₁ → MK-4 conversion is tissue specific (Thijssen and Drittij-Reijnders 1994). Menadione is not a natural form of VK, although in avian and mammalian tissues it can be alkylated to MK-4. This synthetic vitamer has been used as the primary source of VK in animal feeds. High performance liquid chromatography (HPLC) is the method of choice for assessing the different K vitamers in biological matrices (e.g. plasma, tissues/organs, foods) (Davidson and Sadowski 1997). The chemical structures of the principal K vitamers are illustrated in figure 1.

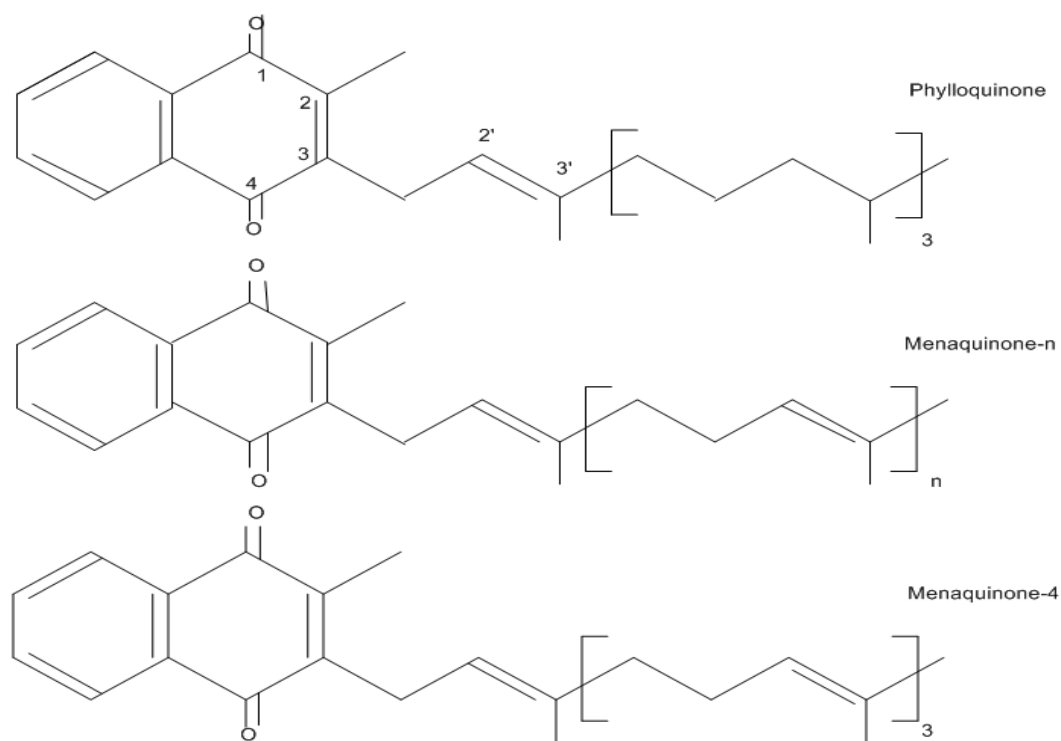


Figure 1. Chemical structures of principal K vitamers (Ferland 2012a).

1.2. Dietary Sources of vitamin K

Phylloquinone from plants is the main dietary source of VK in North America and Europe (Shearer and Newman 2008). Green leafy vegetables contain the highest amount of phylloquinone and contribute 40-50% of total daily intake (Booth, Pennington et al. 1996). Phylloquinone content of swiss chard, spinach and kale has been assessed at more than 300 $\mu\text{g}/100\text{ g}$, while broccoli, brussels sprouts, and cabbage contain 100-200 $\mu\text{g}/100\text{ g}$. Vegetable oils such as soybean, olive, and canola contain significant amounts of

phylloquinone varying from 50-200 $\mu\text{g}/100\text{ g}$ (Shearer, Bach et al. 1996; Booth and Suttie 1998; Ferland G, Nutrition Foundation. et al. 2011). Mixed dishes prepared with phylloquinone-rich oils contribute approximately 15% of total dietary phylloquinone intake (Booth and Suttie 1998).

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The menaquinones are present in limited amounts in foods, however, low levels of menaquinones have been reported in animal products such as chicken egg yolk and butter (Hirauchi, Sakano et al. 1989). Organ livers (especially pig and bovine) are a good source of long chain menaquinones (MK-6 to MK-13) (Koivu-Tikkanen, Ollilainen et al. 2000) as cheeses with MK-8 and MK-9 content have been estimated at between 5 to 20 $\mu\text{g}/100\text{ g}$ (Shearer, Bach et al. 1996). Meat, eggs, and dairy foods are also good sources of MK-4 (Elder, Haytowitz et al. 2006) while MK-7 is found in the highest quantity in fermented soybean products such as Natto, a traditional Japanese dish (Schurgers and Vermeer 2000).

The US Dietary Reference Intakes for VK in 2001 recommended 120 and 90 $\mu\text{g}/\text{day}$ for adult males and females, respectively (Food and Nutrition Board 2001).

1.3. Metabolism

1.3.1. Absorption

In the intestine, VK is incorporated into micelles in a pathway which depends on bile salts and pancreatic juices (Yong Ji 2009).

The efficiency of absorption depends on the dietary source of phylloquinone and the presence of fat in the intestine. The absorption efficiency of phylloquinone in healthy adults is estimated at ~ 80% when administered in its free form (Shearer, Bach et al. 1996; Garber, Binkley et al. 1999) but decreases to ~10% when the vitamin is absorbed from green vegetables (Gijssbers, Jie et al. 1996). In one report, the absorption of phylloquinone from spinach was found to be 4-17% (Gijssbers, Jie et al. 1996; Garber, Binkley et al. 1999); similar low absorption efficiency has been reported for kale (Novotny, Kurilich et al. 2010). Reason for the poor bioavailability of phylloquinone in green vegetables is linked to its tight binding to the chloroplasts (Newman and Shearer 1998).

Adding oil to the vegetables has been shown to increase phylloquinone bioavailability (Booth, Lichtenstein et al. 2002). In one study, adding butter to spinach resulted in a 3-fold (8% to 26%) increase in phylloquinone absorption (Gijssbers, Jie et al. 1996).

Recent isotopic studies further indicate that phylloquinone absorption is affected by meal components (Jones, Bluck et al. 2008). In their report Jones *et al.* (Jones, Bluck et al. 2009) showed that absorption of ¹³C-phylloquinone was 3 times higher in a fast foods type meal in which 80% of phylloquinone came from oil, than in meals in which 80-90% of the phylloquinone came from vegetables.

1.3.2. Transport

In human, > 50% of phylloquinone is carried in triacylglycerol-rich lipoprotein (TRGLP), the rest being carried equally in low (LDL) and high (HDL) density lipoprotein fractions (~ 15% each) (Lamon-Fava, Sadowski et al. 1998; Schurgers and Vermeer 2002; Erkkila, Lichtenstein et al. 2004). In contrast, the menaquinones are mainly transported in the TRGLP and LDL fractions (Schurgers and Vermeer 2002). Phylloquinone circulates in blood in very small concentrations (0.25-2.7 nmol/L) and has been shown to be strongly linked to circulating triacylglycerols (Sadowski, Hood et al. 1989; Azharuddin, O'Reilly et al. 2007).

13.3. Tissue Store and Distribution

Liver is the main storage site for VK, the long chain menaquinones representing the major VK constituent (Suttie 2009). When assessed in humans, the menaquinones were indeed shown to represent ~ 90% and phylloquinone ~10% of total hepatic VK (Suttie 1995). Phylloquinone stores are generally unstable and can decrease up to 25% within 3 days in conditions of dietary depletion (Usui, Tanimura et al. 1990). It is known from animal studies that the K vitamers (notably phylloquinone and MK-4) are present in non-hepatic tissues (Thijssen and Driittij-Reijnders 1994). Specifically, phylloquinone contents in heart and pancreas are higher than in liver while lower concentrations are observed in brain, kidney, salivary glands and lung. Menaquinone-4 is also widely distributed with low concentration in the liver and high levels in brain, pancreas and salivary glands (Thijssen and Driittij-Reijnders 1994; Thijssen, Driittij-Reijnders et al. 1996). The origin of MK-4 in the brain is discussed in more detail in a later section.

Phylloquinone and menaquinones tissue concentration have been shown to increase as a function of diet in a tissue-dependent manner (Thijssen, Driittij-Reijnders et al. 1996;

Davidson, Foley et al. 1998; Ronden, Thijssen et al. 1998). In a study by Ronden *et al.*, phylloquinone and MK-4 concentrations increased in all tissues following ingestion of a phylloquinone or MK-4 supplemented diet, while the ratio between phylloquinone and MK-4 varied among the tissues; the highest values related to the liver and heart. High levels of MK-4 were observed in the pancreas and testis as a result of exclusive phylloquinone diet (Ronden, Thijssen et al. 1998).

Tissue VK level is also affected by age and gender. In a study by Huber *et al.* phylloquinone and menaquinones concentration were assessed in different tissues of male and female rats at 3-, 12- and 24-months old. Except in hearts of 3-months of age, phylloquinone concentrations were not affected by gender. In contrast, MK-4 levels in kidney, heart and brain were significantly higher in females than in males. It was also reported that the hepatic phylloquinone and MK-6 concentration elevated with age; while MK-4 level did not change. In the brain and the extrahepatic tissues of males and females rats, MK-4 concentration declined with age (Huber, Davidson et al. 1999). In humans, plasma phylloquinone concentration has been shown to decrease with age (Sadowski, Hood et al. 1989). Another study investigated the gender differences in relation to VK intake and reported the reduced concentration of phylloquinone and MK-4 in the liver of male and female; however supplemented- phylloquinone diet was associated with higher MK-4 in female rats (Huber, Davidson et al. 1999).

1.3.4. Turnover

Phylloquinone and menaquinone are excreted by a degradative pathway in which the polyisoprenoid side chain is cleaved off into two major carboxylic acid metabolites with 7- and 5-carbon side chains. In humans, around 20% of metabolized phylloquinone is excreted in the urine and 40% to 50% in the feces. Urinary metabolites contain

predominantly glucuronide conjugates of derivatives with an oxidized form of phytyl side chain (Shearer, Mallinson et al. 1972).

Compared with other fat-soluble vitamins, the total body pool of VK is very small and the stored VK in liver is depleted rapidly as a result of restricted dietary VK. The turnover of phylloquinone has been estimated at around 1.5 days (Olson, Chao et al. 2002). The consumed phylloquinone in plasma peaks after 6-9 hours and returns to the baseline within 24 hours (Erkkila, Lichtenstein et al. 2004). It was also shown that urinary VK metabolites decrease by 20% within 15 days as a result of restricted diet (11 μ g/d) and increases rapidly following a rich VK diet (206 μ g/d) (Harrington, Booth et al. 2007). However, long-chain menaquinones remain in circulation much longer i.e. ~ 72 hours in the case of MK-7 (Schurgers and Vermeer 2000).

1.3.5. Transformation of Phylloquinone to MK-4

It is now well-established that tissue MK-4 results from phylloquinone conversion with menadione as an intermediate (Thijssen, Vervoort et al. 2006). Two routes have been suggested: 1) the release of menadione from phylloquinone by removal of the phytyl side chain in the intestine followed by prenylation of menadione in tissues, or 2) the side-chain cleavage of phylloquinone and generation of menadione followed prenylation occurring within the target cell (Ansell, Hirsh et al. 2008). Recently, this team identified the human enzyme responsible for MK-4 biosynthesis. This enzyme is UBiA prenyltransferase domain-containing protein 1 (UBIAD1) which is expressed in numerous tissues in mice and is located in the endoplasmic reticulum (Nakagawa, Hirota et al. 2010) (**Figure 2**).

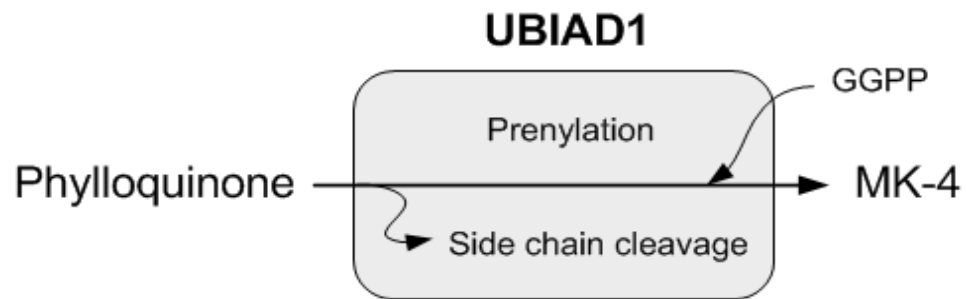


Figure 2. Conversion of phylloquinone to MK-4 by UBIAD1 (Nakagawa, Hirota et al. 2010)

1.4. Vitamin K Dependent Carboxylation

Within the cell, VK acts as a cofactor in the posttranslation of Gla from Glu which is a common residue in all vitamin K dependent proteins (VKDP). The carboxylation is catalyzed by GGCX which is a microsomal enzyme located at the luminal surface of the endoplasmic reticulum. This reaction requires the reduced form of VK, hydroquinone (KH₂), and the presence of carbon dioxide and oxygen (Dowd, Ham et al. 1995). The conversion of Gla to Glu increases the affinity of these proteins for calcium, a characteristic that allows them to bind to biological components associated to their specific role. To convert Glu to Gla residues, the carboxylase uses the energy of VK hydroquinone oxygenation, and this carboxylation is facilitated by a carboxylase recognition signal propeptide (Berkner 2008). In the course of the catalytic sequence, hydroquinone is oxidized to the epoxide form, an inactive form of VK. The vitamin K 2,3-epoxide (KO) is then recycled to quinone and hydroquinone by the VK oxido-reductase (VKOR) which is dependent on the presence of dithiol cofactors (Suttie 2009). Coumarin derivatives such as

warfarin block the action of VKOR (Wallin and Martin 1985). The formation of KO and its conversion back to KH₂ makes the VK cycle (**Figure 3**).

The liver has a unique reductase enzyme which is not sensitive to coumadin drugs. This enzyme is NAD(P)H dependent and makes possible the reduction of the quinone form of VK to KH₂ in the presence of Coumadin drugs, when a high tissue concentration of VK is available (Berkner and Runge 2004) .

In a condition of insufficient dietary VK or the presence of anticoagulant drugs, the carboxylation action is blocked and undercarboxylated forms of proteins are released into the plasma. These inactive forms of proteins are called PIVKAs (protein induced by VK absence or antagonism) and used as a marker of the nutritional status of VK (Lee, Chung et al. 2010) .

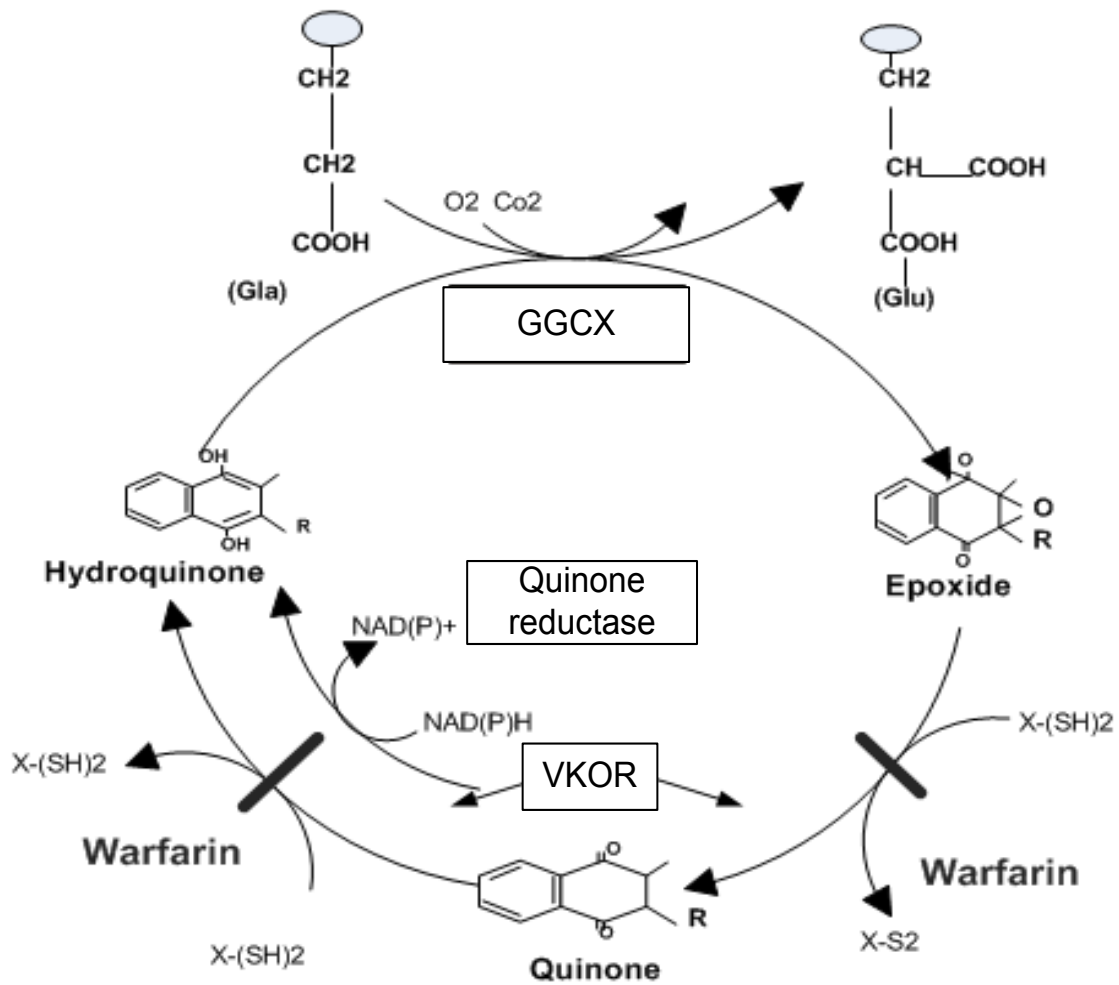


Figure 3. Scheme showing the vitamin K cycle (Ferland G, Nutrition Foundation. et al. 2011)

1.5. Vitamin K Dependent Proteins

Although the discovery of VK in 1930 was associated with its specific role in blood coagulation, the discovery of Gla, a new amino acid common to all VKDP, extended the physiological role of VK. These proteins are involved in bone and calcium metabolism and the brain. The VKDP and their function are summarized in **Table 1**.

1.5.1. Blood Coagulation Proteins

Prothrombine (factor II), factors VII, IX, and X, protein C, S and Z are the VKDPs involved in clotting blood. They are all synthesized in the liver and contain 10-12 Gla residues (Versteeg, Heemskerk et al. 2013). Calcium mediates the binding of the complexes via the Gla residues to the negatively charged phospholipid surfaces expressed by platelets and endothelial cells of the injured cells. The generation of thrombin from prothrombin leads to the formation of fibrin by an activated factor X, which itself is activated by factors VII and IX respectively. Proteins C, S and Z act as the coagulation inhibitors. Protein C inhibits coagulation by deactivating factor Va and VIIIa and elevating the level of fibrinolysis with protein S as a cofactor, while protein Z acts as a cofactor for blocking the activity of factor Xa by protein Z-dependent protease inhibitor (Ferland 1998; Norris 2003). (Davie 2003; Berkner and Runge 2004).

Beside their hemostatic functions the coagulation proteins possess cell signaling activities and are involved in a wide-range of cellular events. For instance, thrombin has been involved in such phenomenon as tumor growth and metastasis, angiogenesis, atherosclerosis, inflammation, survival of neutrophils and monocytes (Sokolova and Reiser 2008; Chen and Dorling 2009). Similarly, protein C has been shown to possess anti-inflammatory and anti-apoptotic properties (Danese, Vetrano et al. 2010)

1.5.2. Bone Proteins

Three main Gla proteins involved in bone metabolism are osteocalcin, matrix Gla proteins (MGP) and protein S (*Ferland G, Nutrition Foundation. et al. 2011*). Osteocalcin is a VKDP synthesized by osteoblasts and odontoblasts, and acts as a negative regulator of bone formation (Ducy, Desbois et al. 1996; Boskey, Gadaleta et al. 1998). It contains 3 Glu residues which increase affinity binding to hydroxyapatite in bone (Booth 2009). In vertebrate species, osteocalcin accounts for around 15-20% of the non-collagenous bone protein, and its molecular weight is 5,8 kDa (Price, Otsuka et al. 1976). Approximately 20% of the newly synthesized bone proteins are secreted in blood circulation and are used as biochemical markers for bone formation. Osteocalcin deficient rats by warfarin administration had excessive bone mineralization and also premature closure of the growth plate (Price 1988). Moreover, it was shown that mice with eliminated osteocalcin gene coding had increased bone mass and improved bone functional qualities (Ducy, Desbois et al. 1996). These data suggest that osteocalcin regulates bone formation negatively.

Although MGP only accumulates in calcified tissues, it is expressed in many soft tissues such as vascular smooth muscle cells. Its main physiological role in vascular calcification is explained in the following section (Luo, Ducy et al. 1997).

Typically protein S is known because of its role in blood coagulation; however, it plays a role in maintaining bone homeostasis as well. In early 1990, two cases of osteopenia were reported in protein S deficient children (Pan, Gomperts et al. 1990). Protein S is synthesized and secreted by osteoblasts and increases the bone resorbing activity of mature osteoclasts, an action which is associated to the ability of protein to bind tyrosine kinase receptors (Maillard, Berruyer et al. 1992; Nakamura, Hakeda et al. 1998).

1.5.3. Protein Involved in Vascular Calcification

MGP contains 5 Gla residue and has a molecular weight around 9.6 kDa (Price, Urist et al. 1983). Unlike osteocalcin this protein is widely distributed in body tissues i.e. lung, heart, kidney and spleen (Fraser and Price 1988). It is also expressed in cartilage (Yagami, Suh et al. 1999) and vascular smooth muscle (Shanahan and Weissberg 1998). MGP acts as an inhibitor of tissue calcification (Fraser and Price 1988; Luo, Ducy et al. 1997; Shearer 2000). A study on mice that targeted the deletion of the MPG gene resulted in mortality of the mice within two months as a result of extensive arterial calcification that led to blood-vessel rupture (Luo, Ducy et al. 1997). Similar results with a lower degree of severity was observed in rats treated with high doses of warfarin (Price, Faus et al. 1998). In humans, mutations of the MGP gene results to Keutel syndrome a condition characterized by abnormal cartilage and arterial calcification (Hur, Raymond et al. 2005). Although the mechanism of action of MGP in vascular calcification is not fully elucidated (Proudfoot and Shanahan 2006), it potentially acts by binding to calcium ions and inhibiting crystal growth (Roy and Nishimoto 2002), and modulating the action of bone morphometric protein type 2 and 4 (Zebboudj, Shin et al. 2003; Yao, Zebboudj et al. 2006).

1.5.4. Other Vitamin K Dependent Proteins

Gas6. In 1993, the VKDP *Gas6* was discovered as a product of the growth arrest-specific gene 6. It has a molecular weight of 75 kDa and contains 11-12 Gla residues; its 673 amino acids shows 43-44% homology with protein S (Manfioletti, Brancolini et al. 1993). *Gas 6* is expressed in numerous tissues and is involved in cellular processes such as cell differentiation, proliferation and activation, phagocytosis and protection against apoptosis (Bellido-Martin and de Frutos 2008). As discussed later, this protein has many actions in the nervous system.

Transmembrane Gla proteins (TMGs). These proteins include proline-rich Gla proteins 1 and 2 (PRGP 1, PRGP2), and TMG3, TMG4. These single-pass integral membrane proteins are widely distributed and although their physiological functions are presently unknown, their chemical structures suggest that they could be involved in cell transduction (Kulman, Harris et al. 1997; Kulman, Harris et al. 2001).

Gla rich protein (GRP). This protein was identified from calcified cartilage in 2008. It has a molecular weight of 10.2 kDa and contains 16 Gla residues. GRP is widely distributed in tissues, but its expression is highest in cartilage. The functions of GRP are not clear at present, but it could regulate calcium in the extracellular environment (Viegas, Simes et al. 2008; Viegas, Cavaco et al. 2009).

Periostin. This protein which is associated with the extra-cellular matrix and is involved in cell migration and angiogenesis was recently identified as a VKDP (Coutu, Wu et al. 2008).

Transthyretin. Identified for its association with thyroid hormones and retinol-binding protein; the role of its Gla residues is presently unknown (Ruggeberg, Horn et al. 2008).

Table 1. The vitamin K dependent proteins and their functions (Ferland G, Nutrition Foundation. et al. 2011).

<i>Protein Category</i>	<i>Physiological function</i>	
Blood coagulation	Hemostasis	Cell signaling activity
Prothrombin	Procoagulant	Thrombin: Platelet aggregation; tumor growth and metastasis; angiogenesis; atherosclerosis and inflammation; cell survival : chemotaxis
Factor VII	Procoagulant	Pro-inflammatory action
Factor IX	Procoagulant	Pro-inflammatory action
Factor X	Procoagulant	
Protein C	Anticoagulant	Anti-inflammatory and anti-apoptotic actions
Protein Z	Anticoagulant	
Protein S	Anticoagulant	Anti-inflammatory and anti-apoptotic actions; phagocytosis of apoptotic cells; mitogenesis (VSMC); neuronal protection
Bone Protein		
Osteocalcin	Negative regulator of bone formation	
Matrix Gla protein	Inhibitor of calcification	
Protein S	Undetermined	
Cell regulation proteins		
Gas 6	Cell differentiation, proliferation, adhesion, and chemotaxis; phagocytosis and anti-apoptosis	
Others		
TMGs	Undetermined	
GRPs	Undetermined	
Periostin	Cell migration and angiogenesis	
Transthyretin	Ligand for thyroid hormones and retinol-binding protein	

2. Vitamin K and Brain Function

2.1. K Vitamers in Brain

The role of VK in brain function has not been systematically investigated even though VK is widely expressed in brain. It is well established that MK-4 is the predominant form of VK in the brain (Thijssen, Drittij-Reijnders et al. 1996). MK-4 concentration in six months old Sprague-Dawley rats has been shown to account for more than 98% of total cerebral VK and both phylloquinone, and MK-4 increase in brain as a result of dietary VK and are affected by age and sex (section 1.3.3). In rats, MK-4 differs among the different brain regions, the highest concentrations being observed in myelinated regions such as pons medulla and midbrain (Carrie, Portoukalian et al. 2004).

2.2. Vitamin K Dependents Proteins in the Brain

Gas6 and protein S are two VKDPs in the brain. They are not directly involved in cognition, although their signalling actions in neurons (Gas6 and protein S), the glia (Gas6) as well as antithrombic function (protein S), suggest contributions of these proteins to the cognitive process (reviewd in Ferland 2012a).

2.2.1. Gas6

The distribution of protein Gas6 was assessed in the central nervous system of rats using biochemical and histological techniques (Prieto, Weber et al. 1999). In the rat embryo, Gas6 expression is mainly in non-neuronal tissues while in the late embryonic stages and during adulthood it is more widely expressed. In the brain of adult rats, Gas6 is expressed in cerebral and piriform cortex, hippocampus, thalamic and hypothalamic structures, midbrain, and cerebellum. It is also highly expressed in the large neurons of the dorsal root ganglia and the neurons of the spinal cord (Li, Chen et al. 1996; Prieto, Weber

et al. 1999). Compared to other VKDPs such as prothrombin and protein S, plasma Gas6 concentrations are much lower (Garcia de Frutos, Alim et al. 1994); concentrations of 0.25 nM (Balogh, Hafizi et al. 2005) or less (Gibot, Massin et al. 2007) having been published. In rats, Gas6 concentrations have also been shown to decrease with age. When investigated in 6-, 12-, and 24-mo-old Fisher 344 rats, Gas6 expression in 24-mo rats was decreased by > 84% in the frontal cortex and by 55% in the striatum and hippocampus when compared to those aged 6 months (Tsaïoun 2000).

Gas6 acts as a ligand for the receptor tyrosine kinases of TAM family (Varnum, Young et al. 1995). In the nervous system, Gas6 has been shown to participate in cell survival, chemotaxis, mitogenesis, cell growth, and myelination. Specifically, Gas6 has been shown to prevent apoptosis of gonadotropin-releasing hormone neurons from undergoing serum deprivation (Allen, Zeng et al. 1999) and of neurons subjected to phospholipase A2-IIA (Yagami, Ueda et al. 2003). Protein Gas6 has also been shown to protect cortical neurons from amyloid β (A β) protein-induced apoptosis. Applying Gas6 to rat cortical neuron cultures resulted in cell apoptosis prevention by inhibiting Ca²⁺ influx and decreasing A β -induced apoptotic features (Yagami, Ueda et al. 2002).

In addition to its neuron-related role, protein Gas6 modulates the survival and functions of glial cell, mainly oligodendrocytes, Schwann cells, and microglia. Schwann cells and oligodendrocytes have important roles in neuron myelination in the central and peripheral nervous systems, and in the transmission of the nervous impulses. In the central nervous system, microglia cells are part of the immune effectors involved in tissue homeostasis and repair (Nimmerjahn, Kirchhoff et al. 2005) and possess phagocytic functions, removing pathogens, cellular debris, and apoptotic cells, which amass over time (Binder, Cate et al. 2008). Recent studies have illustrated the role of Gas6-dependent activation of TAM receptors in the survival of glial cells and the modulation of microglial phenotype. In a study, adding Gas6 to the medium of an oligodendrocyte culture of the human fetal cord elevated cell survival (Shankar, O'Guin et al. 2006).

2-2-2. Protein S

Historically known for its role in blood coagulation, protein S is expressed in the central nervous system. Protein S has been identified in the locus coeruleus, choroid plexus, and astrocytes of adult nervous system (Stitt, Conn et al. 1995; Hall, Obin et al. 2002). Protein S mRNA has also been found in pyramidal neurons of the cortex and hippocampus, and granule neurons of the dentate gyrus of rabbit brains (He, Shen et al. 1995).

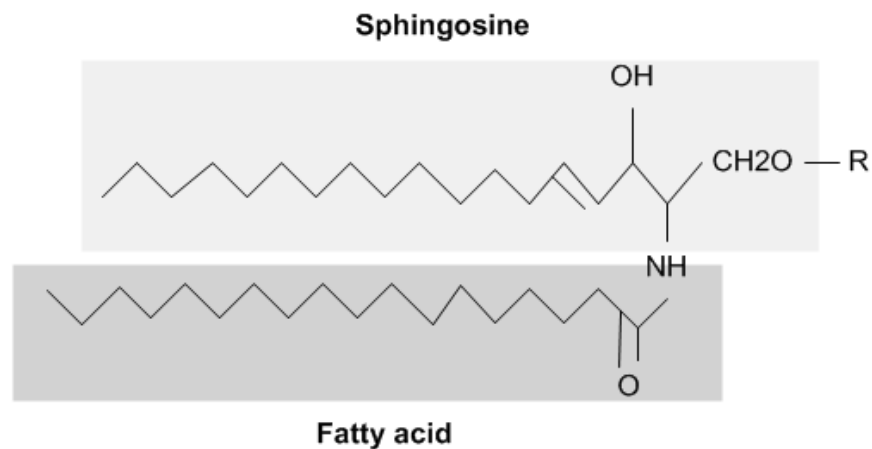
Although the actions of protein S in the brain have been less investigated, it is a ligand for the TAM receptors like Gas 6 (Varnum, Young et al. 1995). *In vivo* and *in vitro*, protein S has been shown to play a role in neuronal protection during ischemic/hypoxic injury. In one study, brain infarction and edema volumes were significantly reduced and post-ischemic cerebral blood flow was improved in mice treated with protein S. Moreover, treatment with protein S resulted in less fibrin deposition and infiltration with neutrophils, less apoptotic neurons and enhanced motor performance (Liu, Guo et al. 2003). In another study, protein S has been shown to protect neurons from N-methyl-D-aspartate-induced toxicity and apoptosis (Zhong, Wang et al. 2010).

3. Vitamin K and Sphingolipids

Sphingolipids are a class of complex lipids found in all mammalian cell membranes. They are abundantly distributed in the cells of the central nervous system in the forms of ceramide, sphingomyelin, cerebroside, sulfatide, and gangliosides (Bartke and Hannun 2009). In addition to their structural function, they are bioactive molecules involved in cell-cell interactions (Ohanian and Ohanian 2001; Chalfant and Spiegel 2005; Siow and Wattenberg 2011; Tani 2011) and participate in important cellular events such as proliferation, differentiation, senescence and transformation (Zeidan and Hannun 2007). Several studies *in vitro* and *in vivo* have demonstrated the contribution of VK in sphingolipid metabolism in the brain (Denisova and Booth 2005).

3.1. The Structure and Metabolism of Sphingolipids

All sphingolipids are composed of a sphingoid long-chain base known as sphingosine that is linked to an acyl chain through an amide bond (**Figure 4**).



Substituent (R)	Sphingolipid
H	Ceramide
Phosphocholine	Sphingomyelin
Sugar (s)	Glycosphingolipid

Figure 4. General sphingolipids structure (Malagarie-Cazenave, Andrieu-Abadie et al. 2002).

The metabolisms of sphingolipids include complex pathways with the sphingoid base subjected to the addition of fatty acids, sugars, or phosphate moieties (Brice and Cowart 2011; Mencarelli and Martinez-Martinez 2012).

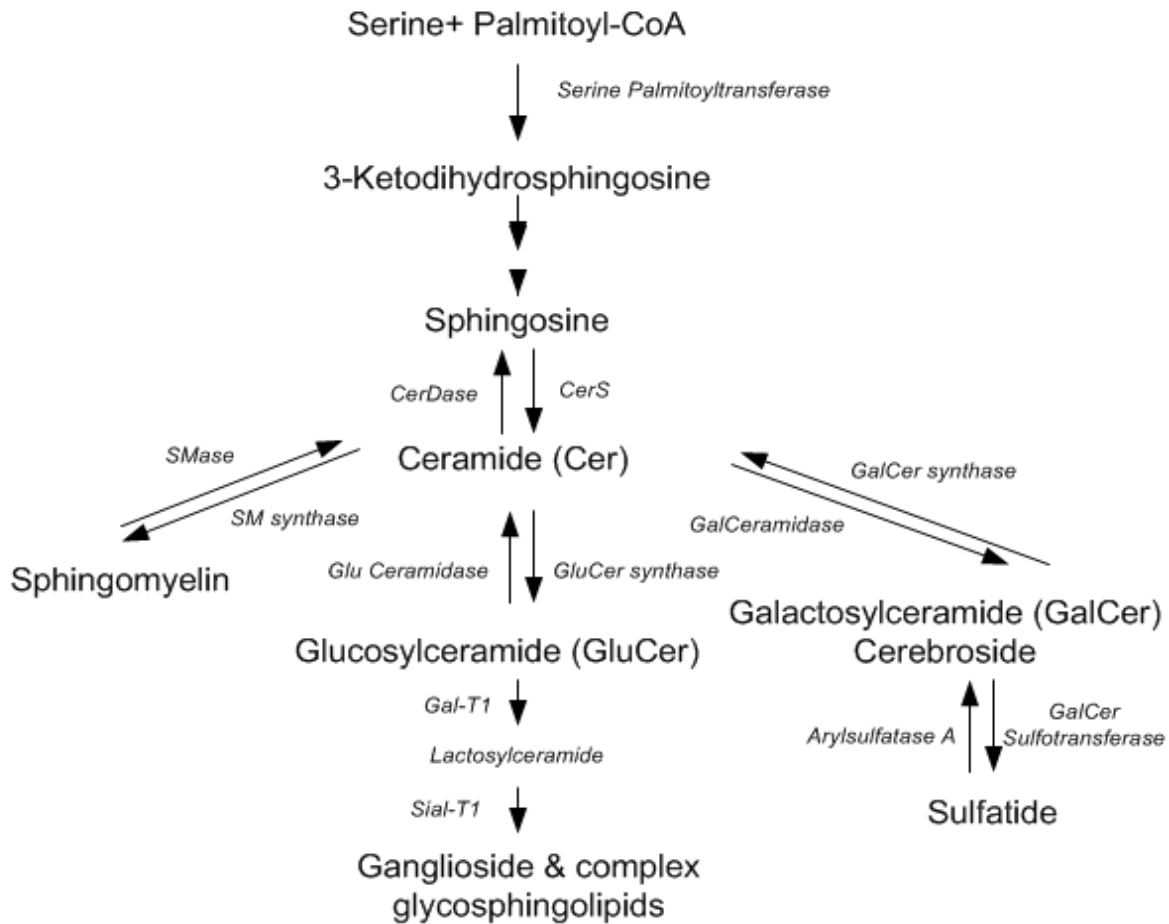


Figure 5. Scheme showing the biosynthesis and metabolic interactions of sphingolipids (Ferland 2012b).

As illustrated in figure 5, the formation of ceramide represents the initial reaction of sphingolipids metabolism and is catalyzed by serine palmitoyl transferase and the combination of serine and palmitoyl-COA. Ceramides can also be generated by the action of sphingomyelinase from sphingomyelin (Mullen, Hannun et al. 2012).

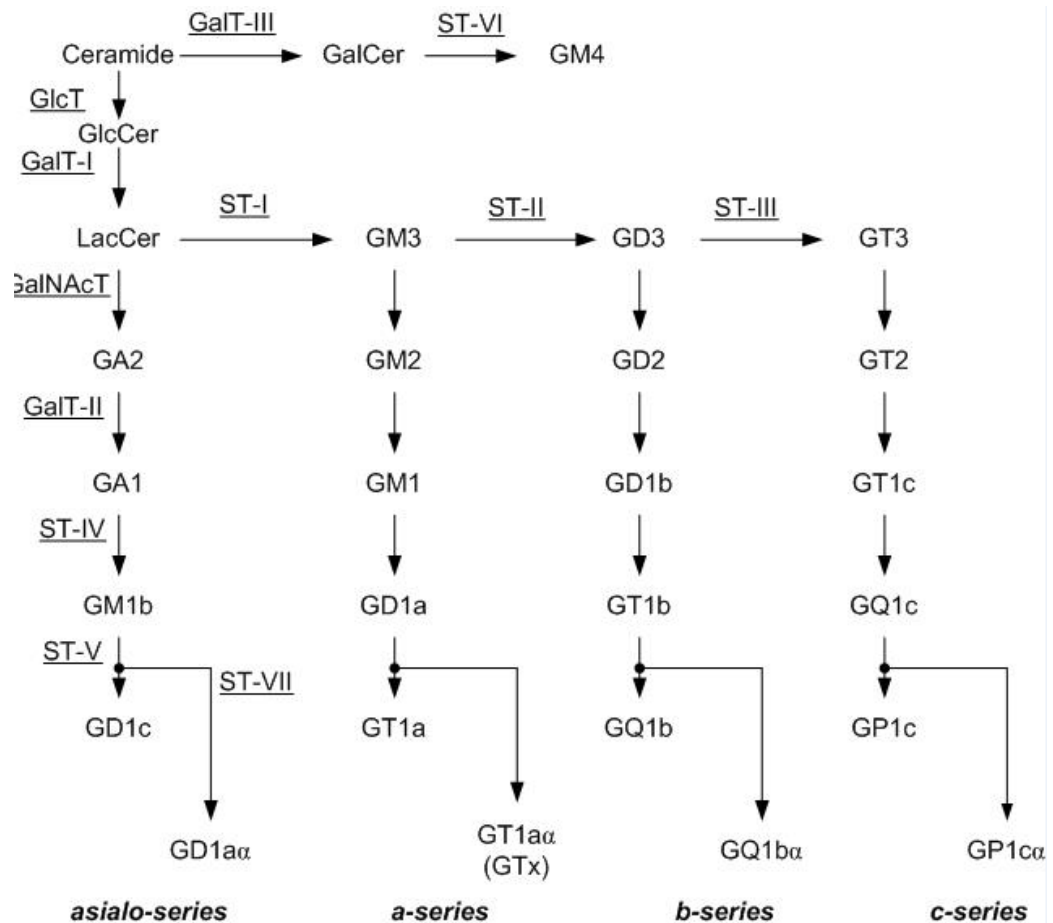
Sphingomyelin, the major sphingolipid in the plasma membrane, is synthesized in the Golgi apparatus by the action of two isoforms of sphingomyelin synthase (SMS1 and

SMS2). The generation of sphingomyelin occurs by transferring a phosphocholine head group from phosphatidylcholine to ceramide (Milhas, Clarke et al. 2010).

Similarly to sphingomyelin, the glycosphingolipids synthesis occurs in the lumen of the Golgi apparatus. Glycosphingolipids are distributed abundantly in the neuronal cells. They are structurally and biosynthetically derived from lactosylceramide, except GM4, which is a major component of myelin and derived from galactosylceramide. The biosynthetic pathway of the ganglio-series of ganglioside is illustrated in figure 6. Lactosylceramide and the hematosides GM3, GD3 and GT3 are used as precursors for complex gangliosides: the asylio- a-, b-, and c-series respectively. In the successive reactions, carbohydrate- and sialic acid residues are attached to glycosyl acceptors by specific glycosyl transferases.

Initially, GM3 is synthesized by adding a sialic acid to LacCer by the action of CMP-sialic acid: Lactosylceramide α 2-3 sialyltransferase. GD3 and GT3 are synthesized by sequential sialic acid addition to GM3, and GD3 by CMP-sialic acid: GM3 α -8 sialyltransferase. The conversion of simple ganglioside to a complex group is catalyzed by UDP-GalNAc:LacCer/GM3/GD3/GT3 β 1-4 N-acetylgalactosaminyltransferase, UDP-Gal:GA2/GM2/GD2/GT2 β 1-3 galactosyltransferase, CMP-sialic acid:GA1/GM1/GD1b/GT1c α 2-8 sialyltransferase (Sandhoff and Kolter 2003; Yu, Tsai et al. 2011; Yu, Tsai et al. 2012).

In the early stages of embryonic brain development, simple gangliosides such as GM3 and GD3 are highly expressed, while in later stages the expression of complex gangliosides such as GM1, GD1a, GD1b, and GT1b are up-regulated (Ngamukote, Yanagisawa et al. 2007; Yu, Nakatani et al. 2009).



Figur 6. Biosynthetic pathways of ganglio-series gangliosides (Yu, Tsai et al. 2012).

Galactosylceramide or cerebroside is formed by the condensation of ceramide and UDP-galactose via the action of the enzyme galactosyltransferase. Moreover, galactosylceramide is a precursor for sulfatide synthesis. Sulfotransferase is the enzyme responsible for the synthesis of sulfatide from galactoceramide by activation of a sulfate of phosphadenosine-5-phosphosulfate. Galactosylceramide and its subsequent metabolite, sulfatide, are highly enriched in myelinated regions of the central nervous system. Galactosyltransferase is located in the endoplasmic reticulum transmembrane and its catalytic site is at the lumen of the endoplasmic reticulum (Gault, Obeid et al. 2010).

Most sphingolipids are degraded in lysosomes and to a lesser extent in the plasma membrane. Sphingolipids are catabolized to ceramide, sphingosine, and sphingosine-1-phosphate via the action of the ceramidase. After ceramide is catalyzed into sphingosine, the conversion of sphingosine to sphingosine-1-phosphate occurs by sphingosine kinases. In the final step of sphingolipid degradation, sphingosine-1-phosphate is catalyzed by sphingosine-1-phosphate lyase function in the endoplasmic reticulum to generate hexadecenal and phosphoethanolamine (Gault, Obeid et al. 2010). Byproducts of sphingolipids catabolism, i.e. sphingosine, can then access the cytosol, and through the salvage pathways regenerate ceramide (Zeidan and Hannun 2007).

In human cells, the breakdown of sphingomyelin is a major part of membrane homeostasis. Sphingomyelin hydrolysis results in ceramide and free phosphocholine via the action of the sphingomyelinase enzyme (Gault, Obeid et al. 2010).

Glycosphingolipid catabolism occurs in the acidic compartments of the cells, the endosomes and the lysosomes. Cellular glycolipids are degraded into their building blocks by the digestion of cellular membranes. Parts of the plasma membrane are endocytosed and transferred through the endosomal compartment to the lysosome. Glycosphingolipids are ultimately cleaved into monosaccharides, sialic acid, fatty acids, and sphingosid bases, which can be either degraded or re-used for sphingolipids biosynthesis (Sandhoff and Kolter 2003).

3.2. The Physiological Function of Sphingolipids

Sphingolipids are important components of the cell membranes. Beside their structural role, they have signalling activities and are involved in the regulation of many cellular processes. Here, we review the functions of the main sphingolipids in the nervous system.

3.2.1. Ceramide and Sphingomyeline

Ceramide and sphingomyelin play leading roles in the regulation of cellular events. Ceramide is involved in the regulation of cell growth, differentiation, senescence, necrosis, proliferation, and apoptosis. It has also been shown that the actions of ceramide are through regulation of protein kinase C, raf-1, and the kinase-suppressor of Ras-, notably by changing the phosphorylation level of key substrates (Bartke and Hannun 2009). In a series of studies, ceramide has been shown to inhibit the neuronal survival pathway regulated by phosphatidil-inositol-3-kinase/Akt (Arboleda, Morales et al. 2009), by conveying activity to the caspase-9/caspase-3 pathway (Movsesyan, Yakovlev et al. 2002; Stoica, Movsesyan et al. 2003). Production of ceramide is induced by stimuli such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1, Fas ligand, ionizing radiation, phorbol esters, heat stress, oxidative stress, and chemotherapeutics (Nikolova-Karakashian and Rozenova 2010; Barth, Cabot et al. 2011; Castro, de Almeida et al. 2011; Barth, Gustafson et al. 2012; Li, Gulbins et al. 2012; Martinez, Chen et al. 2012; Pan, Liu et al. 2012; Van Brocklyn and Williams 2012). High levels of ceramide contribute to the inflammation process and generate reactive oxygen species from mitochondria (Ballou, Lauderkind et al. 1996; Jana, Hogan et al. 2009). Furthermore, the transient hydrolysis of sphingomyelin into ceramide has been observed in response to apoptosis-activation of the sphingomyelinases. These enzymes seem to be important for cellular signal transduction (Jana, Hogan et al. 2009; Milhas, Clarke et al. 2010).

3.2.2. Cerebroside and Sulfatide

Myelin is greatly enriched with cerebroside (galactosylceramide) and sulfatide. The critical physiological function of myelin in the brain is that it insulates axons and provides high axon conductivity for nerve impulses. The myelin sheath ensures rapid intercellular communication which is essential for brain functions such as motor control (Sandell and

Peters 2003; Sandvig, Berry et al. 2004). A series of studies showed that mice lacking galactosyltransferase, the enzyme responsible for synthesizing these two myelin lipids, presented thin and unstable myelin with abundant abnormalities (Bosio, Binczek et al. 1996; Coetzee, Fujita et al. 1996; Coetzee, Dupree et al. 1998; Dupree, Girault et al. 1999; Marcus, Dupree et al. 2000). Another study showed that sulfatide is required for myelin membrane maintenance and axon structures, while galactocerebroside is essential for myelin development (Marcus, Honigbaum et al. 2006). Also, a recent study demonstrated an inhibitory role of sulfatide in myelin-associated axon outgrowth (Winzeler, Mandemakers et al. 2011).

Finally, cerebroside and sulfatide play major roles in regulating oligodendrocyte differentiation and survival. During brain development, oligodendrocyte differentiation is critical for reaching the highest level in the elaboration of myelin sheath (Jana, Hogan et al. 2009).

3.2.3. Gangliosides

In the nervous system, gangliosides interact with numerous regulatory proteins (Yu, Nakatani et al. 2009; Furukawa, Ohmi et al. 2011) and are involved in cell survival, proliferation, and differentiation during brain development (Yu, Nakatani et al. 2009). Gangliosides also contribute to the maintenance and repair of nerve tissues (Kittaka, Itoh et al. 2008; Ohmi, Tajima et al. 2009).

GT1b is one of the major ganglioside subtypes in neuronal and synaptic membranes (Viljetic, Labak et al. 2012). It has been shown to influence cellular apoptosis, and to possess neurotoxic functions against dopaminergic neurons. Specifically, cultures of mesencephalic cells deprived of serum were observed to be more susceptible to GT1b-induced neurotoxicity (Chung, Joe et al. 2001). *In vivo*, GT1b injections into the substantia resulted in the death of nigra dopaminergic neurons, including dopaminergic neurons (Ryu, Shin et al. 2002). Recently, evidence was provided that the Akt/GSK-3/tau signalling pathway is involved in the GT1b-mediated neurotoxic actions (Chung, Bok et al. 2010).

GT1b-induced apoptosis also has been reported in non-neuronal cells like thymocytes and keratinocytes (Zhou, Shao et al. 1998).

GD3, the precursor of GT1b, also possesses an apoptotic function. Induction of apoptosis in cultures of cerebellar granule neurons—by switching the growing medium to a medium containing lower concentrations of potassium—was associated with increased levels of GD3 and the enzyme that synthesizes GD3 from GM3. The exogenous addition of GD3 to the culture accelerated neuronal apoptosis however, no alteration was observed by adding GD1a (Melchiorri, Martini et al. 2002).

GD1a acts as a modulator of cell survival and proliferation. When applied exogenously to the FDC-P1 cell line, GD1a enhanced the proliferation of granulocyte-macrophage colony-stimulating factor (Santos, Maia et al. 2011). In a transgenic model for Huntington's disease (HD), the poor performances of mice in the footprint test were associated with lower cerebellar gangliosides and decreased numbers of GD1a-enriched granule cells (Denny, Desplats et al. 2010). Also, brains of mice suffering from Rett syndrome, a neurodevelopmental disorder of the grey matter, have been shown to contain 15% lower concentrations of GD1a in the cerebrum/brainstem, a finding associated with poorer motor performances (Seyfried, Heinecke et al. 2009).

GM3 contributes to normal behaviour. Mice lacking GM3 caused by gene disruption exhibited progressive motor, sensory dysfunctions, and deterioration in spatial learning and memory (Tajima, Egashira et al. 2009; Tajima, Egashira et al. 2010). Niimi *et al.* (Niimi, Nishioka et al. 2011) investigated cognition, motor activity and emotional behaviour of GM-3 knockout mice and found better performances in the Y-maze test, hyperactivity in the motor activity test, and anxiety in the elevated plus maze test. They also exhibited attention-deficit hyperactivity disorder, reduced attention, and increased impulsive behaviour.

Reduced levels of GM1 have been associated with neurodegenerative disorders such as Huntington's disease (HD). Lowered GM1 synthesis in the brain cells of HD transgenic mice and also fibroblasts from HD patients have been suggested as potentially contributing to the higher susceptibility of HD cells to apoptosis (Maglione, Marchi et al. 2010).

3.3. Sphingolipids and Alzheimer's Disease

Alzheimer's disease (AD) is an age-related disorder characterized by the deposition of A β peptide components and extensive neuronal apoptosis in the brain, notably in the hippocampus. Recent studies suggest that disturbance in the lipids composition of neurons cell membranes and alteration in sphingolipids metabolism could have a role in the neuropathological process of AD (Haughey, Bandaru et al. 2010).

Decreased levels of sphingomyelin and elevated concentrations of ceramides have been reported in various brain regions in AD (Mielke, Haughey et al. 2011; Filippov, Song et al. 2012; Mielke, Bandaru et al. 2012).

A significantly higher concentration of ceramides was reported in the brains of patients suffering from AD as compared to the age-matched control group (Satoi, Tomimoto et al. 2005; Filippov, Song et al. 2012). Finally, high levels of ceramides and dihydroceramides were associated with the strong progression in AD, although higher plasma concentrations of sphingomyelin, dihydrosphingomyelin were associated with less progression (Satoi, Tomimoto et al. 2005). Furthermore, higher levels of serum ceramides were associated with an increased risk of AD (Mielke, Bandaru et al. 2012).

Sulfatides are also affected in AD. Sulfatide concentrations were shown to decrease in the white and gray matter in the earliest stage of AD (Pettegrew, Panchalingam et al. 2001; Han, D et al. 2002; Cheng, Xu et al. 2003). Han *et al.* reported that sulfatides content in the gray matter was depleted by up to 93%, and by 58% in the white matter, in all brain regions in subjects with very mild dementia (Han, D et al. 2002). In the nervous system, sulfatide transport and homeostasis is linked to the apolipoprotein E (apo-E) metabolic pathway. Alterations in apoE-mediated sulfatide trafficking has been associated with sulfatide depletion in the early stages of AD (Han 2007; Han 2010)

Lowered levels of total gangliosides and alterations in gangliosides subtypes have been reported in AD (van Echten-Deckert and Walter 2012). Gangliosides form a complex with A β that is termed "GA β " and which accumulates in AD brain (Kakio, Nishimoto et al. 2002; Zou, Kim et al. 2003; Kakio, Yano et al. 2004; Kimura and Yanagisawa 2007;

Okada, Wakabayashi et al. 2007; Yanagisawa 2011). It has been suggested that in AD brains, ganglioside GM3 and GM1 play a role in amyloid neuropathy and the formation of senile plaques. Transgenic mice expressing a mutant amyloid precursor protein have been shown to accumulate GM3 while presenting little GM1 (Oikawa, Yamaguchi et al. 2009; Chan, Oliveira et al. 2012).

3.4. Vitamin K Involvement in Sphingolipids Metabolism

A study conducted in 1958 by Meir Lev's group was the first to provide evidence for the role of VK in sphingolipids metabolism in bacteria. In this study, VK was shown to act as a growth factor for *Bacteroides melaninogenicus* (Lev 1958). In 1972, a lowered synthesis of sphingolipids was shown to be linked to the VK-free medium; adding VK to the medium resulted in the stimulation of sphingolipid metabolism (Lev and Milford 1972). Subsequently, it was shown that applying VK to the VK-depleted *P.levii* raised the activity of serin palmitoyltransferase—the enzyme catalyzes the condensation of palmitoyl-CoA and serine as the first step of sphingolipid synthesis (Lev and Milford 1973).

These findings were confirmed by studies in mice, when warfarin-induced VK deficiency resulted in a 19% decrease in brain serine palmitoyltransferase activity. Also, a general decrease in sphingolipid levels in the brain was observed, with a 42% decrease of sulfatide, a 17% decrease of sphingomyelin, and a 12% decrease of cerebroside. A supplemented VK diet for three days led to a normal 3-ketodihydrospingosine synthesis, increased levels of sulfatide and ganglioside, and a continuous decrease in levels of cerebroside and sphingomyelin (Sundaram and Lev 1988).

The role of VK in sphingolipids was then confirmed by studies in rodents. Warfarin treatment in mice resulted in a 45% reduction in sulfotransferase activity, the enzyme responsible for sulfatide synthesis, while a supplemented phylloquinone diet led to higher sulfotransferase activity (Sundaram and Lev 1990). Regarding the mechanism whereby sulfotransferase enzyme activity is modulated by VK, it was suggested that phylloquinone

(or menadione) + orthophosphate can provide the required enzyme for ATP and VK involvement in enzyme phosphorylation (Sundaram and Lev 1990; Sundaram and Lev 1992). Similarly, studies showed that the concentrations of sulfatide in the brains of mice with VK- deficient diets were 21% lower than control mice fed VK-replete food (Sundaram, Fan et al. 1996). Studies conducted on mice and rats have shown that VK administration in MK-4 form has an impact on sulfatide levels and also on the activity of galactosyl-ceramide sulphate transferase (Sundaram, Fan et al. 1996).

More recently, brain sphingolipids have been shown to correlate with VK status. A study by Carrie *et al.* (Carrie, Portoukalian et al. 2004) reported that MK-4 levels in brain are positively correlated with sphingomyelins and sulfatides content, and negatively correlated with gangliosides. Positive correlations between MK-4 concentrations and sulfatide have also been reported in the hippocampus and cortex of 12- and 24-month-old male Fisher 344 rats (Crivello, Casseus et al. 2010).

A study on 6-, 12-, and 20-month-old rats with different dietary amounts of VK has shown that sulfatides, cerebroside, and sphingomyelin were present in higher concentrations in the pons medulla and midbrain, while ceramides and gangliosides were higher in the striatum and hippocampus. In 20-month-old rats, low dietary VK was associated with higher levels of ceramides in the hippocampus and lower gangliosides in the pons medulla and midbrain compared with rats that received adequate and high VK intake (Carrie, Belanger et al. 2011).

4. Other Functions of Vitamin K and the Brain

In the brain, the K vitamers have actions of their own beside their carboxylation function to the VKDPs. A study showed that phylloquinone and MK-4 could improve neurite outgrowth on PC12D cells; this action is mediated by the protein kinase A and with MAPK signalling pathways. In addition, both K vitamers resulted in elevating nerve growth factor-induced acetylcholinesterase (Tsang and Kamei 2002). These results

confirmed previous studies, which reported that phylloquinone and MK-4 could enhance the survival of cortex, hippocampal, and striatum neuronal cell types at the later stage of embryogenesis (Nakajima, Furukawa et al. 1993). A recent study of primary cultures of oligodendrocyte precursors and immature fetal cortical neurons provided evidence that the K vitamers, particularly MK-4, can inhibit glutathione depletion-mediated oxidative injuries (Li, Lin et al. 2003). This protective effect of MK-4 was insensitive to warfarin treatment suggesting that the action of MK-4 was independent of the VKDPs. Another study confirmed the neuroprotective function of MK-4 in the methylmercury-induced cell death and found a significant association with a reduction in intracellular glutathione (Sakaue, Mori et al. 2011).

5. Vitamin K and Behaviour

Limited animal studies have suggested that VK plays a role in cognition. In 1984, Cocchetto *et al.* reported that VK deficient rats presented lower locomotor activity when subjected to the open field paradigm; the reduction was reported as 25% compared with the control rats (Cocchetto, Miller et al. 1985). Also, warfarin treatment was linked to an alteration in exploratory behaviour—from more to less exploration, when compared to control rats, and walked less in the center of an open field as a parameter of exploratory behaviour. In the Radial arm maze—a sophisticated test to measure activity and short term memory—depleted VK rats made fewer choices compared with control rats. Rats rendered VK deficient by low VK dietary intake had decreased rates of movement, with no alteration in the accuracy of arm selection.

In a recent study, Carrie *et al.* showed that lifetime low VK consumption was associated with cognitive perturbations in old rats (Carrie, Belanger et al. 2011). In this study, 6-, 12-, and 20-month-old rats were fed either a low 80µg/kg, adequate 500 µg/kg, or high 2000 µg/kg phylloquinone throughout their lives. In the Morris water maze paradigm – a test to assess the cognition – 20-month-old rats receiving the low VK diet had a greater

latency to find the submerged platform compared to other groups. In contrast, locomotion and anxiety behavior were not affected at 20-month-old rats; also that diet effects were not observed at 6- and 12- month-old rats.

In humans, fetal exposure to warfarin during the first trimester of pregnancy has been shown to result in physical anomalies such as optic atrophy, dilation of the cerebral ventricles, blindness, microencephaly, and mental retardation (Hall, Pauli et al. 1980; Pauli 1988). A study in patients in the early stage of AD reported that the phylloquinone intake was significantly lower in the patient ($63 \pm 90 \mu\text{g/d}$ vs. $139 \pm 233 \mu\text{g/d}$) when compared with age- and sex-matched controls. Green vegetables, the main dietary source of phylloquinone, was consumed in lower amounts in patients with AD as compared to control subjects (33% vs. 49%) (Presse, Shatenstein et al. 2008).

5.1. Behavioral Assessment

In laboratory rodents, different types of tests are used to determine whether treatments or conditions affect behavioral status. The Morris water maze is widely used to assess learning ability and cognition; while open field and elevated plus maze tests are used to measure locomotion, exploratory and anxiety-related behaviour, respectively (Walsh and Cummins 1976; Walf and Frye 2007; Sharma 2009).

5.1.1. Morris Water Maze Test

In 1984, Richard Morris developed a device known as the “Morris water maze” (MWM) to assess spatial learning and memory in laboratory rats (Morris 1984). This test has been accepted and extensively used by physiologists and pharmacologists. Although the MWM seems simple at first glance, it is a challenging task for animals and is used in the most sophisticated experiments in neurobiology, neuropharmacology and neurocognition to evaluate the impacts of aging, experimental lesions, and drug effects—especially in rodents. Studies of neurodegenerative and neuropsychiatric disorders where

cognition is damaged (i.e. Alzheimer's disease, Parkinson's disease, and schizophrenia) confirm the importance of this test. The MWM involve the acquisition and spatial localization of visual cues which are processed, consolidated, retained, and retrieved to find a relatively small goal (a hidden platform) (Sharma 2009).

The MWM is a large circular pool (diameter: 150 cm) filled approximately halfway with water at room temperature and with a fixed invisible platform, which is submerged below the water surface (~1 cm). The pool is designated with two principal axes by computer software in order to generate a cross imaginary shape. There are then four points at the end of each line: North (N), South (S), East (E), and West (W), and four equal quadrants. The platform is placed in the middle of one of these quadrants. Several objects or images (e.g. circles, squares, and triangles) are located in the testing room or hung on the wall, so rodents are able to use these visual cues for navigating in the maze (**Figure 7**).

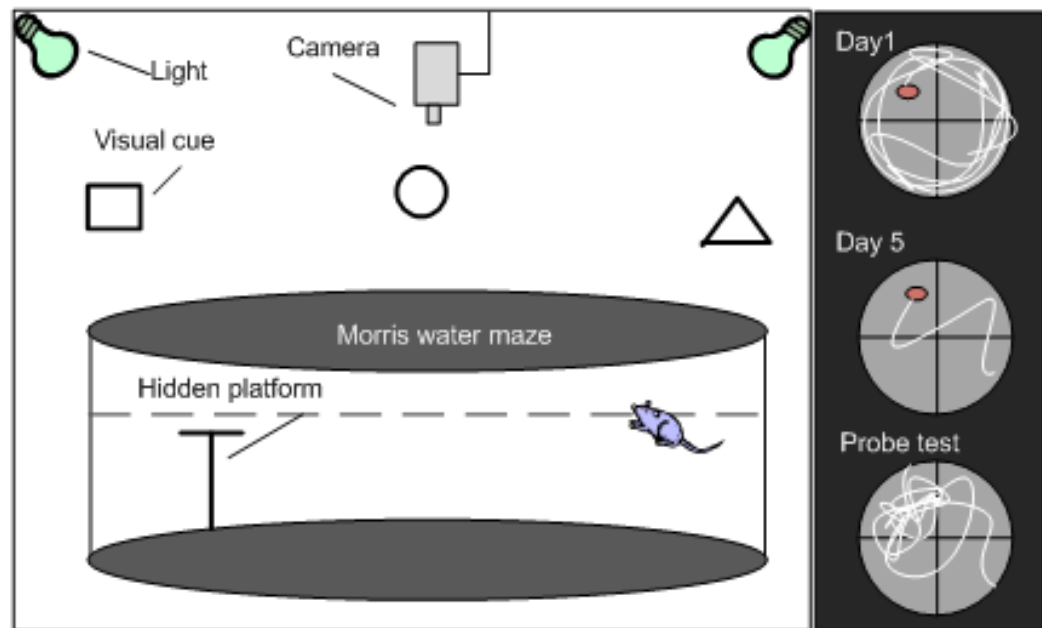


Figure 7. Diagram showing the Morris water maze testing room and apparatus (Alvin V. Terry 2009).

One day after the hidden platform test, a “probe trial” is conducted, meaning that the platform is removed. A rat is placed in the pool without the platform and its performance in the maze is tracked over 30 seconds for two trials. The percentage of time spent in the target quadrant that contained the platform in previous trials is measured. This assessment provides an estimation of accuracy and strength of the rat’s ability to recall the previous platform location.

Immediately after the probe test, rats are subjected to the “cue test” in order to determine the experimental manipulation that leads to the alteration in their visual acuity. In this test, a rat is placed in the pool with a highly visible platform. The time to reach the platform can be assessed with the video and software or the stopwatch (Morris 1984; D’Hooge and De Deyn 2001; Vorhees and Williams 2006; Alvin V. Terry 2009).

5.1.2. Open Field Test

The open field test (OF) is commonly used to assess the general activity and exploratory behavior, in rodents. This test is particularly useful in measurements of the quality and quantity of the activity.

Principally, the OF consists of an enclosure of wood or metal, a plastic square, a rectangularly-shaped arena with surrounding walls in order to prevent escape. The mechanism of assessing the movement involves an overhead tracking video camera and software. The software divides the surface of the box into parallel horizontal and vertical lines (forming a grid) (**Figure 8**).

The activity of the animal during the experiment is tracked via a camera, and the software monitors and computes the number of times that the animal crosses from one box to another on the grid, and also tracks the entries into the center or peripheral squares. The movement is measured by analyzing parameters such as total distance moved, time spent moving, and total crossing squares while the exploratory-related behaviour is assessed by the crosses into the center and time spent in the center. The duration of the experiments in

OF is between 2 and 10 minutes. Short experiment times focus more on exploratory behaviour rather than on activity (Todd D.Gould 2009).

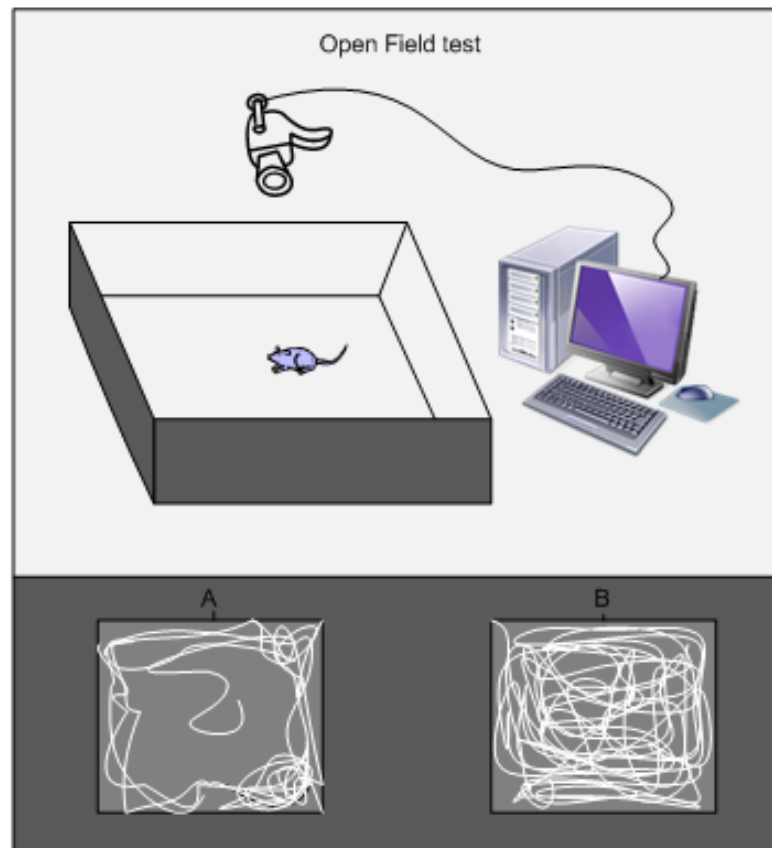


Figure 8. Diagrammatic illustration of the open field arenas and results obtained from open field testing (CJ 2006).

5.1.3. Elevated Plus Maze Test

The Elevated plus maze test (EPM) is commonly used to measure anxiety-like behaviour in laboratory rodents. The EPM test was described for the first time by Montgomery; it was a task with a Y-shaped apparatus including elevated open and closed alleys (Montgomery 1955). In 1984, this task was modified into an elevated maze with a

cross shape, including two open and two enclosed arms, by Handley and Mithani, who described the degree of anxiety by the ratio of time spent on the open arms in relation to the closed arms (Handley and Mithani 1984).

The EPM test has a wide application such as assessing the anxiogenic and anxiolytic effects of a drug, the reproductive effects of senescence/aging and/or exposure to a stressor, and in behavioural assay to investigate the brain sites (e.g., hippocampus, amygdala, dorsal raphe nucleus, etc.) and mechanisms (e.g., GABA, glutamate, serotonin, etc.) underlying anxiety behaviour.

In the EPM test, a rodent is placed in the middle of the intersection of the four arms of the maze, facing the open arms opposite the experimenter. The behaviour of the animal is monitored by a video-tracking system with a computer for five minutes (**Figure 9**).

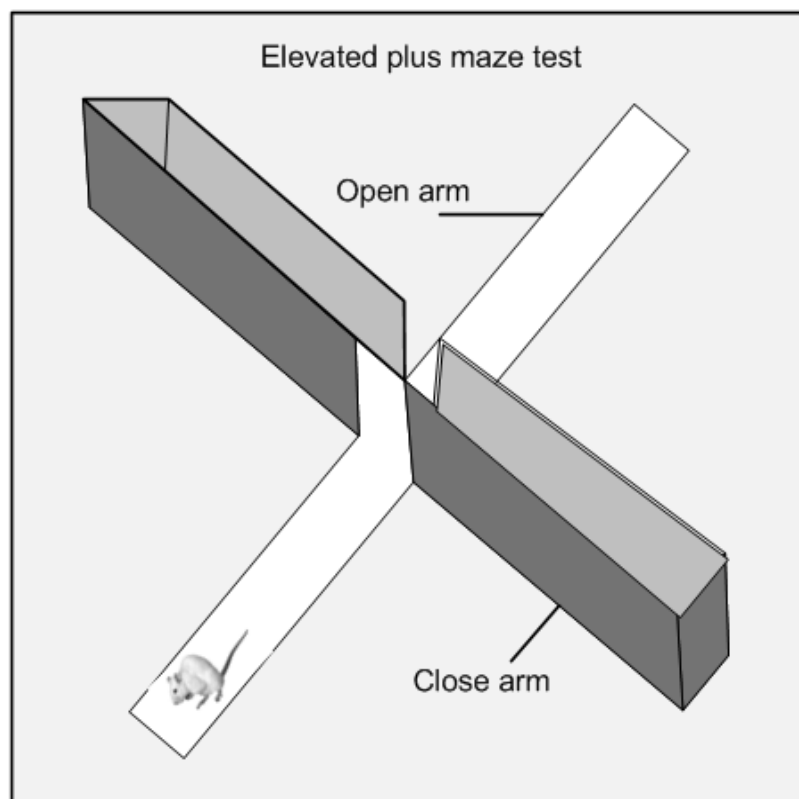


Figure 9. Diagrammatic illustration of the elevated plus maze test

Typically, rodents showed the most robust avoidance-responses being in the open arms during this time. Time spent and entries made on the open and closed arms are the behaviours that are generally recorded in the EPM. The task measures the conflict between the rodent's fear and preference for protected arms versus its innate desire to explore a new environment. Closed arms provide the security whereas the open arms represent exploratory values. When anxious, the animal's tendency is to stay in the enclosed arms rather than in the open, and anxiety-related behaviour is assessed by the degree to which rodents avoid the open arms (Walf and Frye 2007).

6. Physiological Functions of Brain regions

With respect to spatial memory, the hippocampus is the most important region. The hippocampus can integrate information from all over the cortex to build a conjunctive representation. The perirhinal cortex passes information from the ventral visual stream in the inferior temporal cortex into the hippocampus, although the parahippocampal cortex carries the dorsal pathway's spatial information. These inputs merge into the medial entorhinal cortex, and then funnel into the dentate gyrus, CA3, and CA1 of the hippocampus (**Figure 10**) (O'Reilly, Bhattacharyya et al. 2011).

Studies have shown that acquisition of hippocampus-dependent tasks alters neuronal properties, resulting in synaptic transmission. It would be expected that the alteration in synaptic connectivity reflects physiological measures and would be correlated with neuroanatomical characteristics of synaptic contacts (Galvez, Nicholson et al. 2011). It was suggested that the hippocampus plays a critical role in the Morris water maze paradigm—finding the hidden platform—and that the activation of neurons in CA3 hippocampal allows the rodent to locate the submerged platform (Redish and Touretzky 1998). Some studies use the water maze test to demonstrate hippocampal biophysiology plasticity and

reported a reduction in hippocampal afterhyperpolarization (Oh, Kuo et al. 2003; Tombaugh, Rowe et al. 2005).

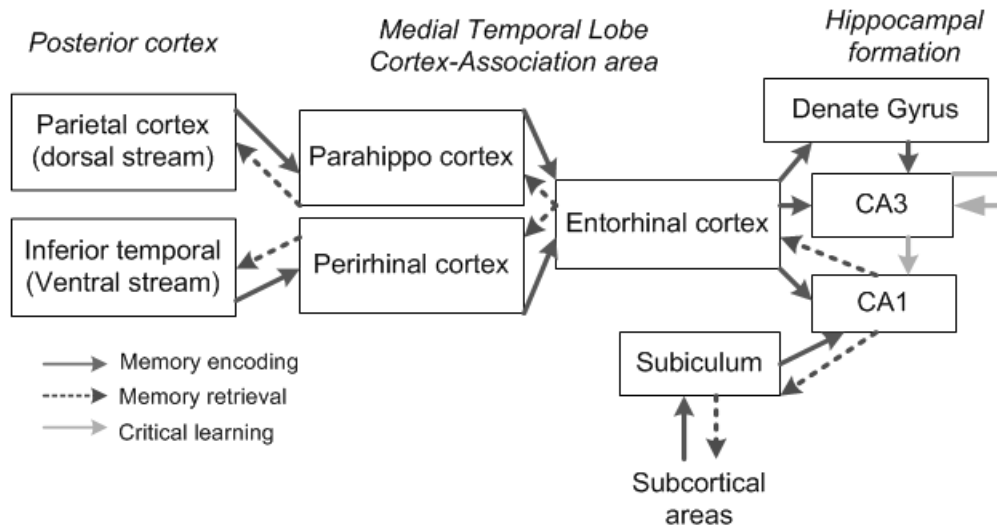


Figure 10. Scheme illustration of the flow of information to be encoded in the hippocampus (O'Reilly, Bhattacharyya et al. 2011)

The cerebellum historically is known for its role in motor control and coordination; however, the cerebellum has a wide-ranging role in cognition, learning and memory, language, and executive function (O'Halloran, Kinsella et al. 2012). Many studies have reported the various functions of the cerebellum such as skill learning, associative learning, working memory, visio-spatial learning, problem solving, spatial memory, orienting of attention, emotional disturbances, and fear conditioning (Cabeza and Nyberg 2000; Paulus, Magnano et al. 2004; Kalashnikova, Zueva et al. 2005; Schmammann and Caplan 2006; Schmammann, Weilburg et al. 2007; Zhu, Scelfo et al. 2007; Schmammann and Pandya 2008; Stoodley and Schmammann 2010). The cerebellum connects to supratentorial regions in two pathways (**Figure 11**): The information flow from the prefrontal cortex reaches to the cerebellum and is involved in the learning process by direct influence on the prefrontal cortex (Kalmbach, Ohyama et al. 2009).

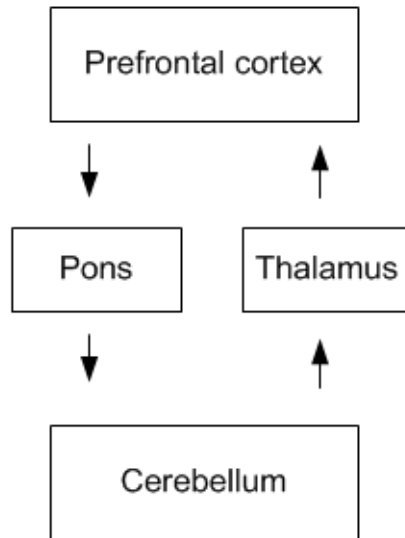


Figure 11. Schematic representation of cerebellum-cerebro pathways (O'Halloran, Kinsella et al. 2012).

The prefrontal cortex is especially important in executive function, and many studies on human and animals demonstrate a wide range of deficits in executive functions as a result of manipulation in this region. Also, the connection of this region to subregions like the amygdala, temporal cortex, thalamus nuclei, hippocampus, and striatum hypothalamus described a complex function mainly contributing to cognitive functions (Kalmbach, Ohyama et al. 2009).

The midbrain as part of the brainstem is mainly known for its roles in planning, initiating, and coordinating the gaze-displacing movements of eyes, head, and body (Gandhi and Katnani 2011), and also in visuomotor targets selection (Port and Wurtz 2009; Nummela and Krauzlis 2010). Recent studies on rats demonstrate the contribution of the midbrain to sensorimotor decision-making (Felsen and Mainen 2012). It was also demonstrated that the forming information of new memories, which is supported by the

hippocampus, is modulated by noradrenergic and dopaminergic system in the midbrain (Sara 2009).

The striatum is involved in the refinement and control of motor movement; however, recent evidence suggests that this brain region contributes to reward- and decision-making, particularly in initiation and action selection (Balleine, Delgado et al. 2007; Haber 2011).

7. Warfarin- Vitamin K Antagonist

Warfarin is prescribed to millions of patients worldwide and has been consistently shown to be highly effective in many clinical settings (Božina 2010).

It has long been used as the main drug in the prevention and treatment of venous thromboembolism e.g. patients with prosthetic heart valves, and in the prevention of myocardial infarction and stroke in high risk individuals. Warfarin and its derivatives decrease blood coagulation by inhibiting the VKOR enzyme required to convert the epoxide form of VK to KH₂ (active form of VK) after it has been involved in the carboxylation of clotting proteins (Described in section 1-4) (Alquwaizani, Buckley et al. 2013)

Dosage of warfarin varies depending on clinical indications, age, and comorbidities. Clinical studies suggest that initial dosing between 2.5 and 10 mg are effective in individuals with arterial fibrillation, venous thromboembolism and myocardial infarction. Initial doses of less than 5 mg are appropriate in the elderly, in patients with impaired nutrition, liver disease, congestive heart failure, and in patients with high risk of bleeding. A starting dose of 2 to 3 mg is usually appropriate for individuals who have undergone heart valve replacement, this lower dosage being due to their higher sensitivity to vitamin K antagonists. The target INR of individuals using warfarin is usually between 2 and 3 (Ageno, Gallus et al. 2012).

In recent years, dietary VK has emerged as an important modulator of long-term anticoagulation stability. Small retrospective studies point to more stable anticoagulation in patients with high VK intakes, and prospective trials have shown that patients receiving small doses of supplemental VK (100-150 µg/d) spend significantly more time in the therapeutic range, require fewer warfarin dose changes, and suffer less bleeding and thrombotic complications over time (Ford and Moll 2008).

To this day, whether a relationship exists between warfarin therapy and cognitive decline has not been investigated.

The Hypothesis and Objectives

In this study, we hypothesized that warfarin-induced VK deficiency will impair cognition and other behavioral domains in rats. Furthermore, this impairment will be associated with an alteration in sphingolipids metabolism in different brain regions.

The principles objectives of this study were:

- To study the effect of warfarin treatment on various behavioral measures (cognition, anxiety, locomotion).
- To study the effect of warfarin treatment on MK-4 distribution in different brain regions.
- To investigate the effect of warfarin on sphingolipids profile in different brain regions.
- To determine whether the behavioral measures are associated with the alteration in sphingolipids profile.

Article

Warfarin-induced vitamin K deficiency is associated with cognitive and behavioral perturbations, and alterations in brain sphingolipids in rats

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Abbreviation used : Vitamin K, VK; MK-4, Menaquinone-4; K₁(25), 2-Methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-naphthalenedione; HPLC, High Performance Liquid Chromatography; HPTLC, High Performance Thin Liquid Chromatography; INR, International Normalized Ratio; UBIAD1, UBiA prenyltransferase domain-containing protein 1;

Key words: Vitamin K; phylloquinone; menaquinone-4; warfarin; cognition; sphingolipids; rat

Abstract

In a previous report, we showed that life-long intake of a low vitamin K (VK) diet was associated with cognitive deficits in old rats. Warfarin is a potent VK antagonist that acts by blocking the VK cycle causing a “*relative VK deficiency*” at the cellular level. This study was conducted to investigate the impact of warfarin on brain with respect to cognition, behavior and brain Menaquinone-4 (MK-4) and sphingolipid status. Fourteen wistar male rats were treated with 14 mg warfarin/kg/d (in drinking water) and subcutaneous VK (85 mg/kg) injection, 3X/week, for 10 weeks; 14 control rats were treated with normal water and injected with saline. Rats were subjected to different behavioral tests after which phylloquinone, MK-4, sphingolipids (cerebroside, sulfatide, sphingomyelin, ceramide and gangliosides), and gangliosides subtypes (GT1b, GD1a, GM1, GD1b) were assessed in various brain regions. Compared to rats from the controls group, those treated with warfarin exhibited longer latencies in the Morris water maze test ($p < 0.05$) as well as hypoactivity and lower exploratory behaviour in the open field test ($p < 0.05$). Warfarin treatment also resulted in a dramatic decrease in MK-4 level in all brain regions ($p < 0.001$), altered sphingolipid concentration, especially in frontal cortex and midbrain ($p < 0.05$), and in a loss of sphingolipid regional differences, notably for gangliosides. Warfarin treatment was associated with lower GD1a in the hippocampus and higher GT1b in the striatum and prefrontal cortex. In conclusion, warfarin-induced VK deficiency alters VK and sphingolipid status in brain with potential detrimental effects on brain functions.

Introduction

Vitamin K is known historically for its role in blood coagulation however, recent studies point to other important functions for this nutrient in the nervous system (reviewed in Ferland 2012a; Ferland 2012b). VK acts as a cofactor for the γ -glutamyl carboxylase enzyme and conveys biological activity to VK-dependent proteins such as Gas6 and protein S (Ferland G, Nutrition Foundation. et al. 2011). In the central nervous system, Gas6 and protein S are involved in a wide range of cellular processes such as cell survival, cell growth, apoptosis, myelination, chemotaxis and mitogenesis (Bellido-Martin and de Frutos 2008).

In brain, VK is also associated with sphingolipid metabolism, a class of complex lipids found in high concentrations in brain cell membranes which includes ceramides, sphingomyelin, cerebroside, sulfatides and gangliosides. In addition to their structural function, sphingolipids have critical roles in cellular events such as proliferation, differentiation, senescence, cell-cell interaction, and transformation. Alterations in sphingolipids metabolism have been observed in the aging process and in neurodegenerative disorders such as Alzheimer and Parkinson diseases (Fantini and Yahi 2010; van Echten-Deckert and Walter 2012). In rodents, brains of mice treated with warfarin, a potent VK antagonist, were shown to contain significantly less sulfatides, sphingomyelin, and cerebroside when compared to those of controls (Sundaram and Lev 1988). More recently, strong correlations were observed between menaquinone-4 (MK-4), the main K vitamer in rat brain, and sulfatide, sphingomyelin, and gangliosides (Carrie, Portoukalian et al. 2004).

There is also data to suggest that VK status can influence cognition and behavior. In a recent study by our team, lifelong low VK consumption was associated with cognitive impairment in old rats, a finding also linked to alterations in sphingolipids in specific brain regions (Carrie, Belanger et al. 2011). In an older study (Cocchetto, Miller et al. 1985),

short term VK deficiency induced through dietary manipulations or warfarin treatment resulted in hypoactivity with no alterations in short-term memory. Furthermore, warfarin administration was associated with a shift from more to less exploratory behaviour. However, in this study, rats' nutritional status was not assessed limiting our ability to link the observed behavioral perturbations to VK status. In light of the fact that warfarin is the most prescribed oral anticoagulant in the world (Božina 2010) and to further characterize its impact on cognition and behavior, we conducted a study in which these components were investigated with respect to MK-4 and sphingolipid status in brain.

Methodology

Rats and treatments

All experimental procedures were approved by the Animal Care Committee of the Université de Montréal according to the guidelines of the Canadian Council on Animal Care. Male Wistar rats (initial body weights of 175 to 250g) were obtained from Charles Rivers Canada. Rats were housed two per cage, kept under a 12 hours reversed light/-dark-cycle and 22°C, and had free access to diets and water. The warfarin-induced VK deficiency protocol used in the present study was that developed by Price *et al.* (Price, Faus *et al.* 1998) and as modified by Moreau *et al.* (Essalihi, Dao *et al.* 2003). Specifically, 14 randomly selected rats were treated with warfarin 14 mg/kg/d in their drinking water and subcutaneous VK (85 mg/kg/d) injections, three times per week for 10 successive weeks. Phylloquinone injections were started one week before warfarin administration and warfarin dosing was adjusted three times per week by checking the drunken volume. Fourteen control rats were treated with normal water and injected with saline. Both groups of rats were fed a diet containing 750 µg phylloquinone/kg diets. Body weights were recorded twice a week, and food consumption was assessed once a week by subtracting the amount of food left over from the amount of food provided the previous week. Blood clotting capacity (international normalized ratio, INR) of warfarin-treated rats was

monitored once a week using blood samples from the tail vein using a Coagucheck device (Roche, Canada). The health of the rats was monitored daily throughout the experimental period.

Behavioural tests

After the treatment period, rats from both groups were subjected to the Morris water maze (Morris 1984), the open field (Todd D.Gould 2009), and the elevated plus maze (Walf and Frye 2007), these tests assessing cognition, motor activity and anxiety, respectively (in light cycle).

Morris water maze

Morris water maze test consisted of a large, circular, metal pool (diameter: 150 cm) filled approximately half-way (30 cm) with 22°C water containing a fixed invisible platform (10 cm²) which was submerged below the water surface (~ 2 cm). Several objects or images (e.g. circles, squares, and triangles) were hung on the walls of the room in which the test was conducted, so the rats could use them as visual stimuli for navigating in the maze. Each day of the trial, rats were released in the water in one of the four quadrants randomly. It took them about 90 sec to find the platform; if successful they were allowed a 30 s stay on the platform. If they did not reach the platform within 90 s, they were physically placed on it and allowed to rest there for 30s. The test was conducted for three trials per day over five consecutive days. The performance of each rat was monitored with a camera mounted above the pool and recorded via a DVD recorder. Latencies to find the platform, swim speed and time spent in each quadrant were obtained and analyzed using a software developed by Dr Rochford (McGill University).

A *Probe trial* was conducted one day after the hidden platform tests to assess spatial accuracy. The platform was removed, and the rats were placed in the pool and allowed to swim freely while their search patterns were tracked over 30 seconds for two trials. The percentage of time spent in the target quadrant where the platform had been located during

the learning trials was computed. This assessment provided an estimation of accuracy and strength of the rats' recalling of the previous platform position.

Immediately after the probe trial, rats were subjected to the "Visible Platform Test" or *Cue trial* to determine if the treatment resulted in alterations in visual acuity. In this test, rats were placed in the pool with a highly visible platform for 30 sec.

Open field

The open field consisted of a black wooden box (60×50×50 cm) with surrounding walls to prevent escape. Assessing the movement of the rats involved an overhead tracking video camera and a tracker system. The software divided the surface of the box into parallel horizontal and vertical lines (forming a grid). After 30 minutes of adaption to the testing room, the rats were placed in the center of the area, and the activity of the animal during the experiment was monitored. Rats were allowed to explore the field freely for five minutes. Motor activity was based on total distance moved (cm) and total number of square crossings, while exploratory behavior was assessed by the animals' number of crossings into the center and the percentage of time spent in the center. This experiment was conducted once a day for three consecutive days and data analyzed by the Field 2020's software (HVS image).

Elevated plus maze

Anxiety-like behavior was measured using elevated plus maze, a test that relies on the rodent's innate fear of open spaces and height. The elevated plus maze consisted of a grey wooden cross with four arms (90×8 cm) that was elevated 70 cm from the floor. Two opposite arms were open, while the other two were enclosed by side end walls (10 cm high). After 30 minutes of adaptation to the room, rats were placed in the middle of the intersection of the four arms facing an open arm and their behavior was recorded for five minutes. The total time spent in the open and closed arms, and the number of open arm

entries and total number of entries were computed. The elevated plus maze test was conducted once (Pellow, Chopin et al. 1985).

Biochemical analyses

After completion of the behavioural tests, rats were subjected to biochemical analyses. Specifically, rats were anesthetized with *pentobarbital* and bled from the abdominal aorta. The brains were gently removed on ice and dissected into midbrain, cerebellum, hippocampus, frontal cortex, striatum, thalamus and sensorimotor cortex. The brain regions were frozen in liquid nitrogen and stored at -80°C until assessments.

Vitamin K analysis

Phylloquinone and MK-4 were quantified by reverse-phase HPLC (n=6-8/treatment group) as previously described (Carrie, Portoukalian et al. 2004). Briefly, tissue samples were grounded in Na₂SO₄ and extracted with acetone containing an internal standard K1(25). The dried extracts were then mixed with water and hexane, and the hexane phase was purified by solid phase extraction on silica gel columns. The quantitative analysis of phylloquinone throughout the text and MK-4 was performed by HPLC using C-18 reverse phase column and fluorescence detection. The calibration standard contained phylloquinone, MK-4, and K1(25) at 2ng in 50 µL.

Sphingolipids analyses

Sphingolipids which included sulfatides, cerebroside, sphingomyelin, ceramides, and gangliosides were quantified (n=6/treatment group) in the various brain regions as described previously (Carrie, Portoukalian et al. 2004). Briefly, brain lipids were extracted with chloroform:methanol (2:1) and separated according to the method of Folch *et al.* (Folch, Lees et al. 1957). Gangliosides were eluted according to the method of Williams and McCluer (Williams and McCluer 1980) and free sialic acids were quantified according

to Jourdian *et al.* (Jourdian, Dean *et al.* 1971) Ceramides, cerebroside, sulfatides, and sphingomyelin were loaded onto LC-NH₂ columns (Supelco) and eluted sequentially. The sulfatide fraction was further applied to C-18 silica columns. All fractions were evaporated and suspended in chloroform: methanol (2:1). Ceramides, cerebroside and sphingomyelin were quantitated by determination of sphingosine with fluorescamine according to Naoi *et al.* (Naoi and Lee 1974) and sulfatides with azure A according to Kean *et al.* (Kean 1968).

Ganglioside analysis

Gangliosides subtypes were analyzed by high-performance thin-layer chromatography (HPTLC) using 20*10cm silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). Purified gangliosides standards (GT1b, GD1a, GM1 and GD1b) were purchased from Metraya.Inc. Each ganglioside standard mixtures were spotted in duplicate on each plate. The HPTLC plates were prewashed with chloroform to eliminate contamination that could affect gangliosides mobility (Ravindranath, Muthugounder *et al.* 2004). After a brief drying period, plates were placed in a chamber containing 200ml of developing solvent [chloroform/methanol/0.25% aqueous CaCl₂ (60/35/7.5 v/v/v)] (Ardail, Popa *et al.* 2003) which had equilibrated for at least 2 hours (Ledeen and Yu 1982). Plates were run for 60 min, air dried, and sprayed with Resorcinol reagent (10ml of 2% resorcinol in water, 40ml concentrated HCl and 0.250 ml of 0.1M copper sulphate)(Svennerholm 1957). Plates were then placed face down on a clean glass cover plate in an oven at 120°C until appearance of the bands (about 20min)(Hauser, Kasperzyk *et al.* 2004). The percent distribution of the individual gangliosides was determined by scanning the HPTLC plates and computing the bands using Image J software (version 1.42, National Institutes of Health, Bethesda, MD). Individual bands from each lane were identified by comparison with a standard mixture.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version5). All data were expressed as means \pm SEM. Body weight, food consumption, Morris water maze and open field test were assessed using repeated-measures ANOVA, with warfarin treatment as the main effect and time as the repeated measure. Cue and probe trial, swimming speed and latencies on individual days (Morris water maze), elevated plus maze, MK-4, sphingolipids concentrations and different subtypes of gangliosides for each brain regions were analyzed by t-test as a function of warfarin effect. The regional distributions of MK-4, sphingolipids concentration, and gangliosides for each group were compared by one-way ANOVA followed by Tukey's post hoc test. Pearson's correlation test was performed to estimate the linear relationship between each class of sphingolipids and MK-4. Differences at $P < 0.05$ were considered as significant.

Results

Body weight and food consumption

Body weights increased in both groups during the experimental period ($P < 0.0001$) although the warfarin-treated rats gained significantly less weight compared to controls ($P = 0.0062$). Also, food intake was lower in rats from the warfarin than the control group ($P = 0.0006$) (**Figure 1**).

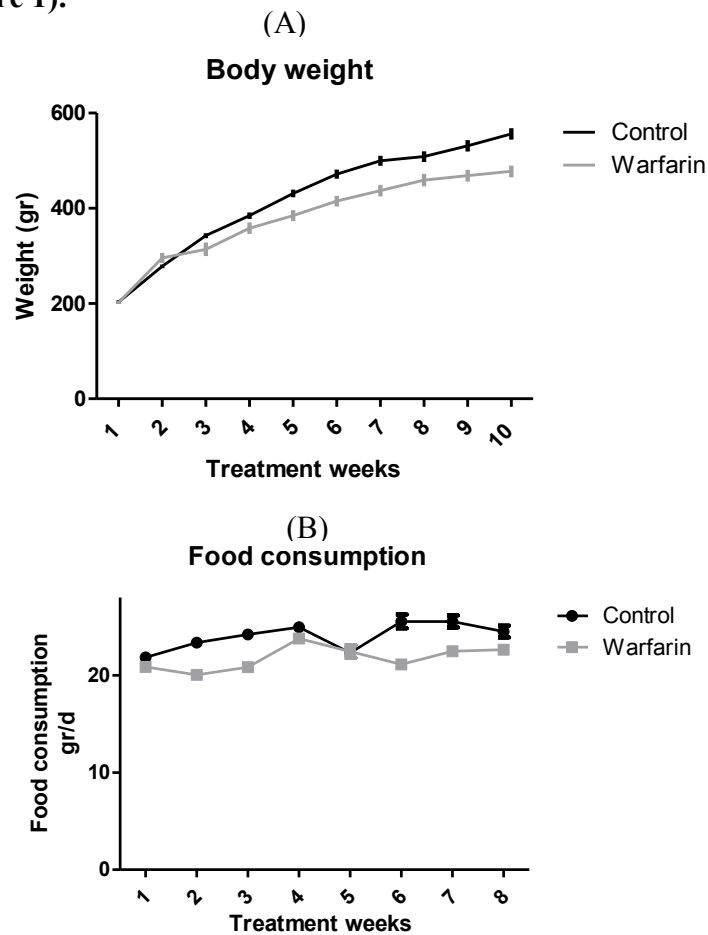


Figure 1. (A) Body weight and (B) food consumption of warfarin and control rats. Values are Mean \pm SEM, $n = 14$. Body weights of rats from the warfarin group differed from those of the control group during the 10-wk treatment period $P = 0.0062$, and they consumed less food $P = 0.0006$.

Behavioral tests

Morris water maze

Time to find the hidden platform decreased across the successive training days for both groups ($P < 0.0001$), suggesting that learning occurred across trials. On day 2, the warfarin-treated rats presented significantly longer latencies when compared to those from the control group ($P = 0.0212$). Warfarin treatment had no effect with respect to the Cue trial ($P = 0.4640$) and swim speed ($P = 0.6267$). In the Probe trial, both groups had a preference for the quadrant in which the platform was located during the learning trials but results were not affected by warfarin treatment ($P = 0.4541$) (**Figure 2**).

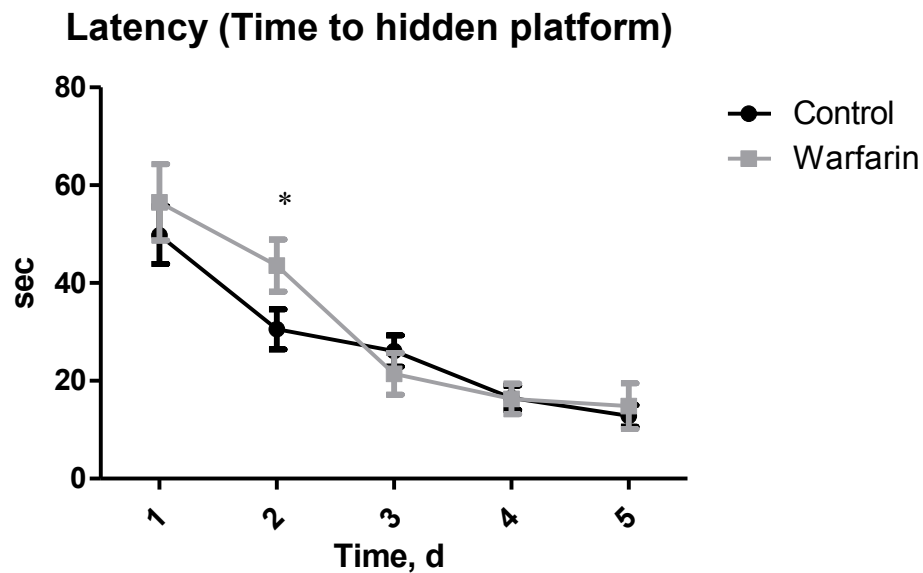


Figure 2. Performance of control and warfarin treated rats in the Morris Water maze test. Values are Mean \pm SEM., $n = 14$. * warfarin group differ from control group $P < 0.05$

Open field

Distance moved ($P=0.0474$) and total number of squares crossed ($P=0.0495$) were reduced in warfarin-treated rats over the three days of trials when compared to control rats. Warfarin treatment also resulted in lower %time spent in the center squares ($P=0.0056$) and number of center square crossings ($P=0.0037$) (**Figure 3**).

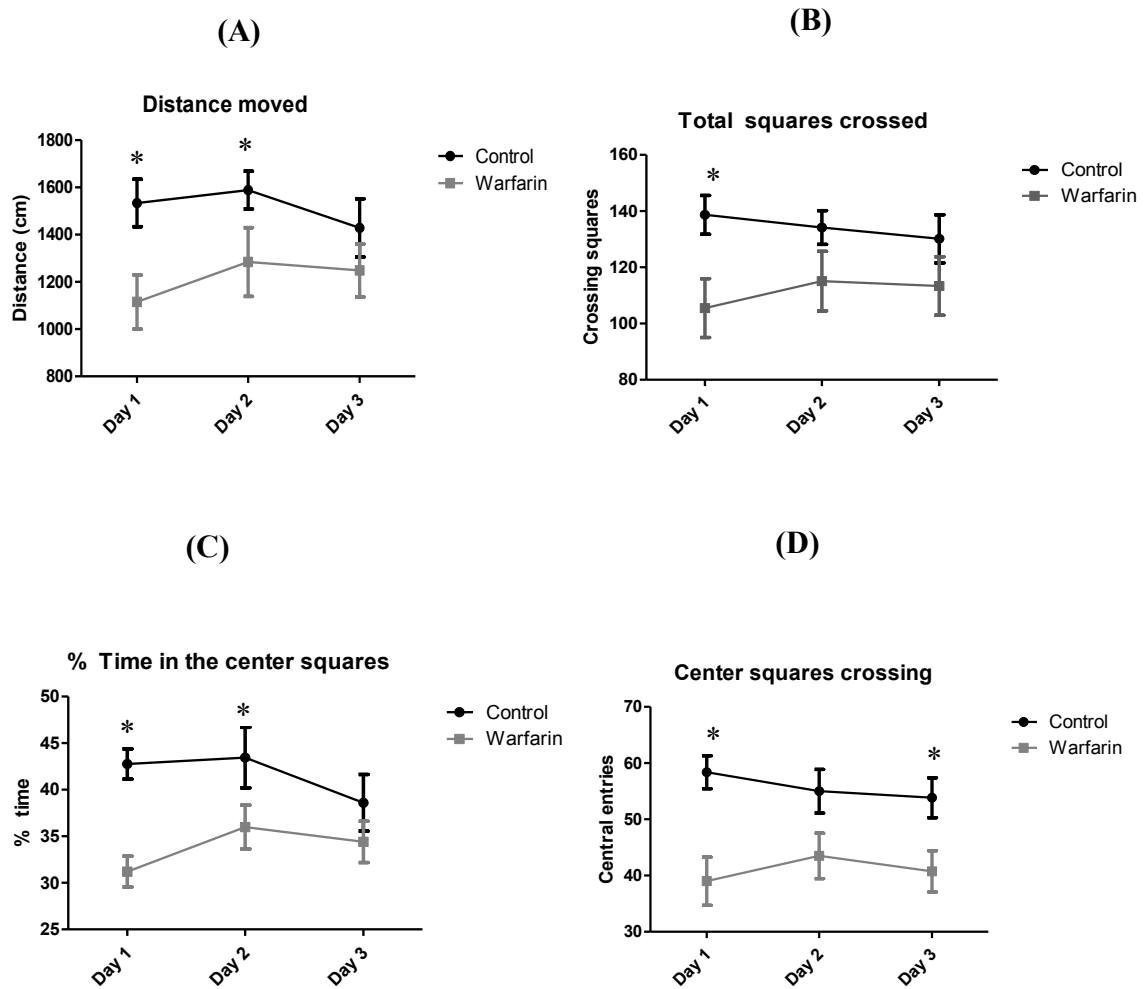


Figure 3. Open field test. (A) Distance moved. (B) Total squares crossed. (C) % time in the center squares. (D) Center squares crossing. The locomotor activity and exploratory capacity of warfarin treated rats were significantly lower than control group ($P < 0.05$).

Elevated plus maze

Time spent in the open arms ($P=0.3560$), % open arms/total time ($P=0.5905$) and percentage of open arms entries / total entries ($P=0.1998$) as a factor of anxiety-like behaviour, did not differ significantly between control and warfarin groups (**Figure 4**).

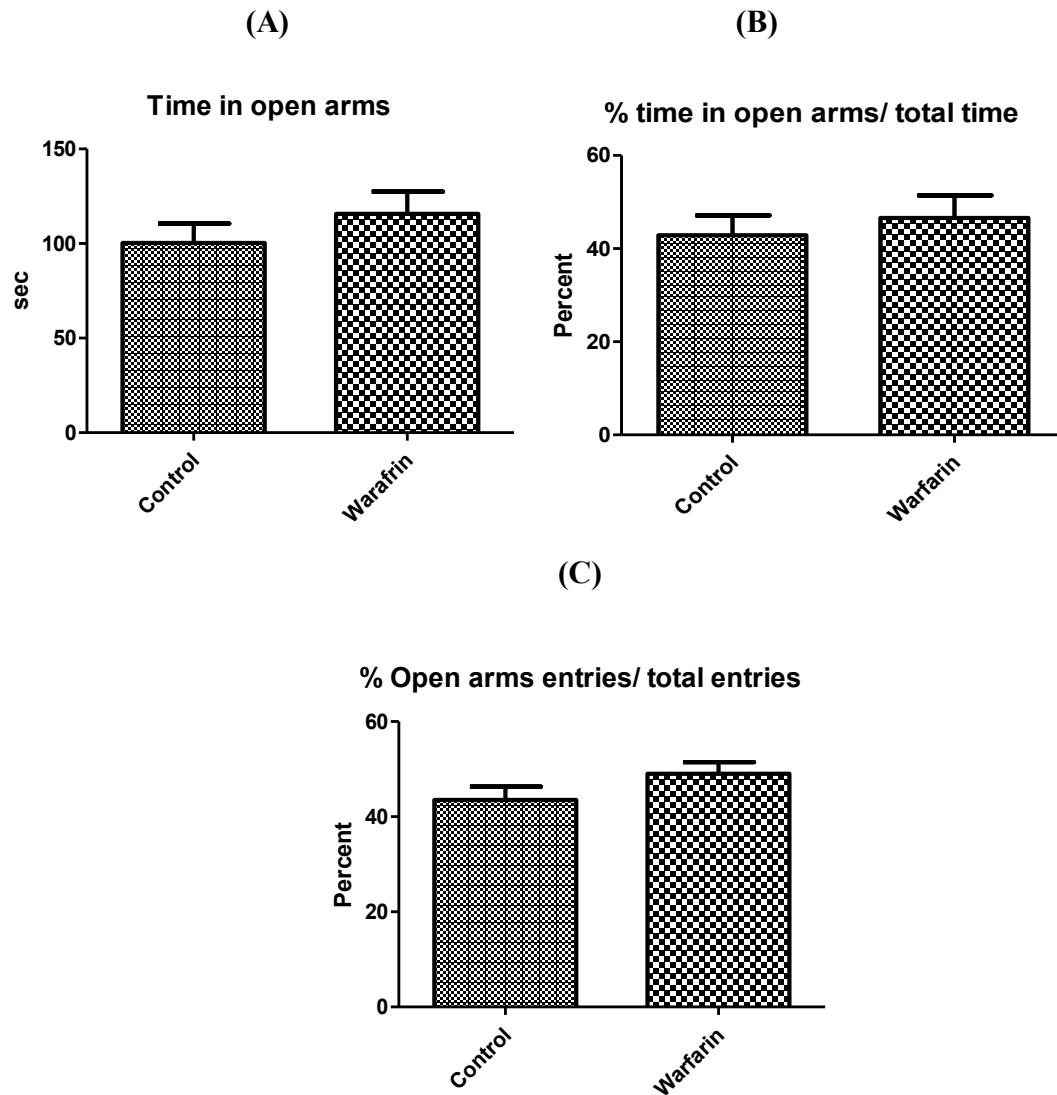


Figure 4. *Elevated plus maze test. (A) Time spent in the open arms (B) Percent time spent in the open arms (C) percentage of open arms entries for control and warfarin group as the indicators of anxiety level. There is no significant difference between the control and warfarin groups ($P>0.05$).*

Biochemical analysis

Vitamin K

MK-4, the major form of VK in brain (~ 86.17% of total VK), decreased dramatically in all brain regions as a result of warfarin treatment ($P < 0.001$) (**Table 1**). MK-4 concentrations in warfarin-treated rats were 25-42% those of controls. In control rats, MK-4 was unevenly distributed in the brain regions ($P = 0.0006$) with the highest concentrations observed in frontal cortex, midbrain, thalamus and sensori-motor cortex, and the lowest concentrations observed in the cerebellum, hippocampus and striatum. Interestingly, these regional differences totally disappeared in brains of the warfarin-treated rats, concentrations of MK-4 being comparable across all brain regions ($P = 0.3822$).

Table 1 MK-4 concentrations in brain regions of control and warfarin treated rats ¹

Regions	Control	Warfarin
(Menaquinone-4) pmol/g		
Striatum	98.48 ± 6.55 ^{a, c, x}	39.49 ± 3.91 ^{a, y}
Hippocampus	99.56 ± 13.70 ^{a, b, c, x}	38.27 ± 3.53 ^{a, y}
Frontal cortex	136.2 ± 9.03 ^{b, c, x}	48.13 ± 4.78 ^{a, y}
Midbrain	143.5 ± 14.04 ^{b, x}	40.55 ± 4.30 ^{a, y}
Sensori-motor cortex	136.3 ± 7.23 ^{b, c, x}	44.89 ± 6.65 ^{a, y}
Cerebellum	88.36 ± 6.39 ^{a, x}	36.11 ± 2.27 ^{a, y}
Thalamus	126.4 ± 9.65 ^{a, b, c, x}	33.59 ± 6.63 ^{a, y}

¹ Values are means ± SEM, n=6-8. Means in a column without a common letter (a, b, c) differ, $P < 0.05$; means in a row for each vitamer without a common letter (x, y) differ, $P < 0.05$

Sphingolipids

Concentrations of each class of sphingolipids varied across brain regions ($P < 0.0001$). In both groups of rats, highest concentration of sulfatide, sphingomyelin and cerebroside were observed in the midbrain ($P < 0.001$) and in control rats, the striatum

contained higher ganglioside concentrations ($P < 0.01$). While in the control group gangliosides varied significantly across brain regions, this pattern disappeared as a result of warfarin treatment (**Table 2**).

Except for sulfatides and gangliosides in the frontal cortex, warfarin administration resulted in a decline in all sphingolipids in most brain regions. Warfarin was associated with a significant decrease in ceramide concentration in all regions of the brain ($P = 0.0378$). The reduction in ceramide was more pronounced in the midbrain (13.29% ; $P = 0.0386$), but also tended to decrease in the striatum (12.24% ; $P = 0.0694$) and frontal cortex (17.79% ; $P = 0.0592$). Also, warfarin administration resulted in a significant decrease in the level of sphingomyelin (38.9% ; $P = 0.0038$), and to a lesser degree, sulfatide (14.0% ; $P = 0.0684$) in the midbrain. In the frontal cortex, however, the concentration of sulfatide and ganglioside increased by 35.8% ($P = 0.0267$) and 25.1% ($P = 0.0101$), respectively.

Table 2 Sphingolipid concentrations in brain regions of control and warfarin treated rats ¹

	Striatum	Hippocampus	Frontal cortex	Midbrain	Sensori-motor Cortex
<i>Cerebrosides, $\mu\text{mol sphingosine/g}$</i>					
Control	15.59 \pm 0.93 ^{a, x}	3.94 \pm 0.36 ^{a, y}	1.84 \pm 0.26 ^{a, y}	27.33 \pm 1.63 ^{a, z}	4.37 \pm 0.39 ^{a, y}
Warfarin	14.29 \pm 0.52 ^{a, x}	5.53 \pm 0.98 ^{a, y}	1.38 \pm 0.10 ^{a, y}	25.00 \pm 1.93 ^{a, z}	4.32 \pm 0.25 ^{a, y}
<i>Sphingomyelin, $\mu\text{mol sphingosine/g}$</i>					
Control	1.75 \pm 0.13 ^{a, x}	0.71 \pm 0.03 ^{a, y}	1.41 \pm 0.07 ^{a, x, y}	4.24 \pm 0.36 ^{a, z}	1.21 \pm 0.04 ^{a, x, y}
Warfarin	1.62 \pm 0.04 ^{a, x}	0.75 \pm 0.06 ^{a, y}	1.55 \pm 0.15 ^{a, x}	2.60 \pm 0.24 ^{b, z}	1.22 \pm 0.03 ^{a, x, y}
<i>Sulfatides, $\mu\text{mol cerebroside sulfate/g}$</i>					
Control	2.80 \pm 0.21 ^{a, x}	1.22 \pm 0.11 ^{a, y}	0.83 \pm 0.07 ^{a, y}	4.41 \pm 0.20 ^{a, z}	1.12 \pm 0.05 ^{a, y}
Warfarin	2.55 \pm 0.13 ^{a, x}	1.22 \pm 0.14 ^{a, y}	1.04 \pm 0.04 ^{b, y}	3.80 \pm 0.23 ^{a, z}	1.20 \pm 0.06 ^{a, y}
<i>Ceramides, $\mu\text{mol sphingosine/g}$</i>					
Control	0.31 \pm 0.01 ^{a, x}	0.25 \pm 0.01 ^{a, y, z}	0.29 \pm 0.02 ^{a, x, y}	0.20 \pm 0.01 ^{a, z}	0.27 \pm 0.01 ^{a, x, y}
Warfarin	0.27 \pm 0.01 ^{a, x}	0.24 \pm 0.02 ^{a, x}	0.23 \pm 0.01 ^{b, x, y}	0.18 \pm 0.01 ^{b, z, y}	0.27 \pm 0.01 ^{a, x}
<i>Gangliosides $\mu\text{mol sialic acid/g}$</i>					
Control	0.72 \pm 0.05 ^{a, x}	0.51 \pm 0.04 ^{a, y}	0.47 \pm 0.02 ^{a, y, z}	0.37 \pm 0.02 ^{a, z}	0.53 \pm 0.03 ^{a, y}
Warfarin	0.63 \pm 0.04 ^{a, x, y}	0.48 \pm 0.04 ^{a, x, y, z}	0.63 \pm 0.05 ^{b, x}	0.40 \pm 0.02 ^{a, z}	0.55 \pm 0.02 ^{a, x, y, z}

¹ Values are means \pm SEM, n=6-8. Means in a column (a, b) or row (x, y, z) with superscripts without a common letter differ, $P < 0.0$.

Correlation between MK-4 and sphingolipids concentration in brain

The associations between MK-4 and the different sphingolipids were investigated in brain for both groups of rats (Figure 5). In control rats, MK-4 was positively correlated with sulfatide ($r=0.2343$, $P=0.03494$) and negatively correlated with gangliosides ($r=-0.5340$, $P=0.0272$). A strong trend for a positive correlation was also observed between MK-4 and sphingomyelin ($r=0.4197$, $P=0.0828$). However, in warfarin-treated rats, no correlations were observed between MK-4 and either of the sphingolipids, indicating a loss of their associations.

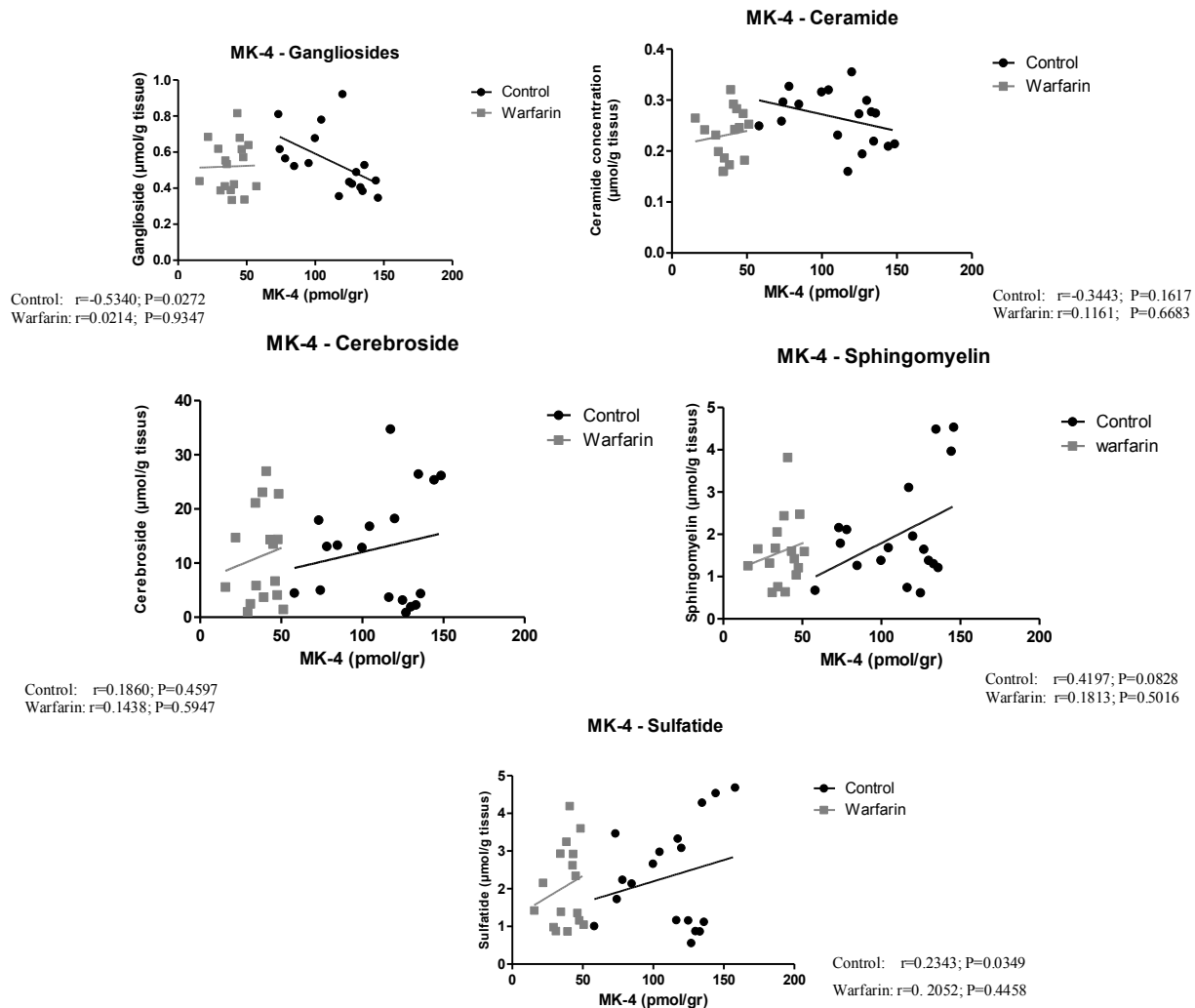


Figure 5. Relationship between brain MK-4 and sphingolipids in control and warfarin rats. Data points are concentrations from 5 brain regions.

HPTLC ganglioside fractions

In both groups of rats, gangliosides subtypes GD1b, GD1a, GT1b and GM1 were differentially distributed across brain regions ($P < 0.0001$) (**Table 3**). Specifically, GD1a was present in highest concentration in the frontal cortex, striatum, hippocampus and sensorimotor cortex while significantly higher levels of GD1b, GT1b and GD1b were observed in the midbrain. In control rats, GM1 was present in higher concentrations in the striatum and midbrain, while in warfarin rats, GM1 levels were higher in the midbrain compared to other regions ($P < 0.05$ in all cases).

Finally, warfarin administration resulted in a significant reduction in GD1a in the hippocampus ($P = 0.0161$), and in significant increases in GT1b in the striatum ($P = 0.0303$) and frontal cortex ($P = 0.0357$).

Table 3 Ganglioside distribution in different brain regions of control and warfarin rats ¹

	Striatum	Hippocampus	Frontal cortex	Midbrain	Sensori-motor cortex
Percentage					
GT1b					
Control	9.16 ± 1.19 ^{a,x}	22.03 ± 0.80 ^{a,y}	21.86 ± 0.53 ^{a,y}	33.96 ± 1.33 ^{a,z}	29.47 ± 1.54 ^{a,z}
Warfarin	15.36 ± 2.22 ^{b,x}	22.94 ± 0.47 ^{a,y}	24.64 ± 0.53 ^{b,y}	30.95 ± 1.75 ^{a,z}	25.91 ± 1.53 ^{a,y,z}
GD1b					
Control	7.23 ± 1.23 ^{a,x}	7.76 ± 0.99 ^{a,x}	7.75 ± 1.31 ^{a,x}	16.63 ± 1.14 ^{a,y}	8.82 ± 0.74 ^{a,x}
Warfarin	8.58 ± 1.02 ^{a,x}	7.90 ± 0.72 ^{a,x}	7.96 ± 0.72 ^{a,x}	16.42 ± 1.30 ^{a,y}	9.03 ± 1.09 ^{a,x}
GD1a					
Control	50.29 ± 2.28 ^{a,x}	49.19 ± 1.70 ^{a,x}	50.60 ± 1.652 ^{a,x}	14.59 ± 0.88 ^{a,y}	39.94 ± 1.65 ^{a,z}
Warfarin	48.30 ± 1.70 ^{a,x}	44.06 ± 0.69 ^{b,x,z}	48.60 ± 0.66 ^{a,x}	17.49 ± 2.03 ^{a,y}	41.27 ± 1.12 ^{a,z}
GM1					
Control	33.30 ± 3.13 ^{a,x}	21.01 ± 1.73 ^{a,y}	19.79 ± 1.62 ^{a,y}	34.81 ± 2.00 ^{a,x}	21.77 ± 1.15 ^{a,y}
Warfarin	27.76 ± 3.01 ^{a,x}	25.10 ± 1.01 ^{a,x,y}	18.80 ± 1.53 ^{a,y}	35.14 ± 1.12 ^{a,z}	23.79 ± 1.45 ^{a,x,y}

¹ Values are means ± SEM, n=7. Means in a column (a, b) or row (x, y, z) with superscripts without a common letter differ, $P < 0.05$.

Discussion

This study provides further evidence that warfarin-induced VK deficiency results in learning deficits, hypoactivity and lower exploratory behaviour in rats, a finding associated with a dramatic decrease in brain MK-4 and an alteration in sphingolipid profile.

Warfarin acts as an inhibitor of the VK oxidoreductase enzyme responsible for the recycling of the vitamin. As a result of warfarin treatment, and as shown in the present study, VK concentrations in the form of MK-4 significantly decrease in the brain. To our knowledge, this study is the first to link cognitive and behaviour impairments to VK status and sphingolipid metabolism.

Specifically, warfarin treatment was associated with longer latencies on day two in the Morris water maze paradigm, a result that could suggest impairment of the encoding component of the learning process. This deficit was not due to visual or motor impairments as results in the cue test and rats' swim speed were not statistically affected by warfarin treatment. Moreover, although prothrombin times were slightly higher in warfarin-treated rats [warfarin 2.69s vs. control 1.04s (P=0.0072)] there were no sign of bleeding in any of the animals. Interestingly, results reported here on day two are comparable to those observed in rats subjected to lifelong low VK consumption (Carrie, Belanger et al. 2011), further suggesting a role for VK in hippocampal function.

In open field test, warfarin-treated rats travelled significantly less distance and crossed fewer squares compared to control rats, suggesting a negative impact of warfarin on locomotion. Also, warfarin administration was associated with decreased exploratory behaviour, rats from the warfarin group spending less time moving through the center squares of the box. However, anxiety-related behaviour was not affected by warfarin administration when rats were subjected to the elevated plus maze test. Our results on locomotion are in line with those reported by Cocchetto *et al.* (Cocchetto, Miller et al. 1985) who observed a significant reduction in locomotor activity as a function of low VK intake, and less exploratory behaviour following warfarin treatment.

In contrast to the Cochetto *et al.* study in which rats' VK status was not investigated, we report that our warfarin treatment lead to a significant reduction of MK-4 concentration in all brain regions. MK-4 is the predominant K vitamer in the brain. It represents more than 98% of total VK in brains of Sprague Dawley rats (Carrie, Portoukalian *et al.* 2004; Carrie, Belanger *et al.* 2011), and in the present study involving Wistar rats, MK-4 accounted for more than 86% of total brain VK. Cerebral MK-4 is currently deemed to be the by-product of phylloquinone conversion (Okano, Shimomura *et al.* 2008). Recently, the human UBIAD prenyltransferase domain-containing protein 1 (UBIAD1) enzyme was identified as responsible for MK-4 synthesis (Nakagawa, Hirota *et al.* 2010). Interestingly, our results suggest that the UBIAD1 enzyme is inhibited by warfarin for despite injections of high doses of phylloquinone MK-4 in brain tissue decreased dramatically.

In the present study, sphingolipids were found to be altered, especially in the midbrain and frontal cortex, as a function of warfarin treatment and depletion of brain MK-4. Specifically, ceramide and sphingomyelin were significantly reduced in the midbrain, results for sulfatides not reaching statistical significance. Ceramides were also significantly decreased in the frontal cortex.

Ceramides make up the backbone structure of all sphingolipids and have been shown to mediate many cellular processes such as differentiation, growth, apoptosis and senescence (Zeidan and Hannun 2007). Similarly, sphingomyelins are involved in cellular signalling and are important regulators of death and growth signals at the plasma membrane (Milhas, Clarke *et al.* 2010; Peter Slotte 2013). Finally, sulfatides contribute to the maintenance of myelin and axon structures (Marcus, Honigbaum *et al.* 2006), and are involved in oligodendrocytes differentiation and myelination (Jana, Hogan *et al.* 2009).

In contrast to what was observed for ceramides and sphingomyelin, warfarin treatment was associated with a significant increase in gangliosides in the frontal cortex. Furthermore, when ganglioside subtypes were investigated, the warfarin-induced increase in frontal cortex was mainly explained by higher levels of GT1b. Increased levels of GT1b

were also observed in the striatum while the hippocampus was found to contain lower levels of GD1a.

In the nervous system, gangliosides interact with numerous regulatory proteins (Furukawa, Ohmi et al. 2011; Yu, Tsai et al. 2012) and participate in cell survival, proliferation, and differentiation during brain development (Yu, Nakatani et al. 2009). Gangliosides also contribute to the maintenance and repair of nerve tissues (Kittaka, Itoh et al. 2008; Ohmi, Tajima et al. 2009). In the human brain, approximately half of the sialic acid is carried on gangliosides GD1a and GT1b (Sturgill, Aoki et al. 2012). It has been shown that GT1b modulate cellular apoptosis and possess neurotoxic functions against dopaminergic neurons. Specifically, cultures of mesencephalic cells deprived of serum were observed to be more susceptible to GT1b-induced neurotoxicity (Chung, Joe et al. 2001). *In vivo*, GT1b injections into the substantia resulted in the death of nigral neurons, including dopaminergic neurons (Ryu, Shin et al. 2002). GT1b-induced apoptosis also has been reported in non-neuronal cells like thymocytes and keratinocytes (Zhou, Shao et al. 1998).

In contrast to the actions of GT1b, GD1a acts as a modulator of cell survival and proliferation. When applied exogenously to FDC-P1 cell line, GD1a enhanced the proliferation of granulocyte-macrophage colony-stimulating factor (Santos, Maia et al. 2011). In a transgenic model for Huntington's disease, the poor performances of mice in the footprint test were associated with lower cerebellar gangliosides and decreased numbers of GD1a-enriched granule cells (Denny, Desplats et al. 2010). Also, brains of mice suffering from Rett syndrome, a neurodevelopmental disorder of the grey matter, have been shown to contain 15% lower concentrations of GD1a in the cerebrum/brainstem, a finding associated with poorer motor performances (Seyfried, Heinecke et al. 2009).

Given the reported biological actions of GT1b and GD1a, the observed higher levels of GT1b in the striatum and frontal cortex, and lower levels of GD1a in the hippocampus could have contributed to the poor performance of the warfarin-treated rats in the Morris water maze test as these regions are directly linked to the cognitive process. The hippocampus is the most important brain region with respect to spatial memory (O'Reilly,

Bhattacharyya et al. 2011) whereas the prefrontal cortex is especially important in executive function (Chudasama 2011). The striatum is involved in the control of motor movement although recent evidence suggests that this brain region is also involved in reward and decision making (Balleine, Delgado et al. 2007; Haber 2011).

Finally, warfarin treatment as tested in the present study resulted in a loss of correlation between MK-4 and the different class of sphingolipids. In control wistar rats, and as had been observed in Sprague-Dawley rats, Carrie *et al.* (Carrie, Portoukalian et al. 2004) MK-4 was positively correlated with sphingomyelin, sulfatide, and negatively correlated with gangliosides. In contrast, no correlations were observed in warfarin-treated rats between MK-4 and either of the sphingolipids, indicating a loss of their associations.

In conclusion, we found that warfarin-induced VK deficiency is associated with cognitive impairment, hypoactivity and lower exploratory behaviour in rats. These findings were associated with a dramatic reduction of brain MK-4, and an alteration in the sphingolipids, notably the gangliosides. In light of the actions of gangliosides GT1b and GD1a, their altered profile in regions involved in cognition (hippocampus) and locomotion (striatum) could have contributed to results of this study. Additional studies are needed to determine whether warfarin doses used in the clinical settings represent a cognitive risk for patients treated with this drug.

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Discussion

The present study investigated the role of VK in relation to behaviour and its potential link to the sphingolipids profile in rat brains that were subjected to warfarin treatment.

In the brain, warfarin blocks the VK oxidoreductase enzyme, which is responsible for the recycling of the vitamin, and leads to VK deficiency. Unfortunately, despite the wide usage of warfarin as an oral anticoagulant drug, there has been no pertinent study on its impact on cognition. To our knowledge, this study is the first to link cognitive and behaviour impairments to VK status and sphingolipids metabolism.

In this study we found that warfarin treatment was associated with higher latencies in day two of the Morris water maze test, suggesting an impairment of the encoding component of the learning process. This learning deficit on day two was similar to what was observed in our previous study, when rats were fed lifelong low VK diets (Carrie, Belanger et al. 2011). Also, the learning deficit was not due to visual or motor ability impairments, for neither performance in the cue test nor swim speed were affected by warfarin administration. Importantly, there were no signs of hemorrhage in any of the animals despite slightly higher prothrombin times in warfarin-treated rats [warfarin 2.69s vs. control 1.04s (P=0.0072)].

Furthermore, motor activity and exploratory behaviour declined as a function of warfarin treatment when rats were subjected to the open field test. Warfarin-treated rats traveled significantly lower distances and crossed fewer squares in the open field compared to control rats, suggesting lower activity as a function of warfarin treatment. Also, warfarin administration was associated with alterations in exploratory behaviour; rats from the warfarin group crossed fewer central squares and spent less time moving through the center of the box. However, anxiety-related behaviour—assessed using the elevated plus maze test—was not affected by warfarin administration. These findings concord with results from a study by Cocchetto *et al.* (Cocchetto, Miller et al. 1985) who reported a significant reduction in locomotor activity by low VK dietary intake, and lower exploratory behaviour when rats were rendered VK deficient by warfarin treatment. In Cocchetto *et al.* study, the

concentration of VK content in the brain was not investigated. Our study found that warfarin treatment was associated with a dramatic reduction of MK-4 concentration in all regions of the brain; MK-4 concentrations in warfarin group were 60-70% less than in control.

MK-4 is the predominant K vitamer in the brain. It represents more than 98% of the total VK in brains of Sprague Dawley rats (Carrie, Portoukalian et al. 2004; Carrie, Belanger et al. 2011), and more than 86% of the total brain VK of Wistar rats as observed in the present study. It is well established that cerebral MK-4 is produced by phylloquinone conversion (Okano, Shimomura et al. 2008). Recently, the human UBIAD1 enzyme was identified as responsible for MK-4 synthesis (Nakagawa, Hirota et al. 2010). Our results suggest that the UBIAD1 enzyme is inhibited by warfarin: although high doses of phylloquinone were administered to the rats, it was not converted to MK-4 in brain tissue. However, the role played by warfarin in the enzymatic activity of UBIAD1 in the transformation of phylloquinone to MK-4 needs further investigation.

In the present study, warfarin treatment and MK-4 depletion were associated with an alteration in the sphingolipids profile in the brain. We found that ceramide and sphingomyelin were significantly decreased in the midbrain; there was no statistically significant change in sulfatide levels. Ceramides were also reduced in the frontal cortex. Ceramides—the backbone structure of sphingolipids—have been shown to mediate many cellular processes such as differentiation, growth, apoptosis and senescence (Zeidan and Hannun 2007). Sphingomyelin—as an important sphingolipid in the structure of cell membranes—is involved in cellular signalling (Milhas, Clarke et al. 2010; Peter Slotte 2013). Sulfatides play important roles in the maintenance of myelin and axon structures of the central nervous system (Marcus, Honigbaum et al. 2006), and are involved in the regulation of oligodendrocytes differentiation and myelination (Jana, Hogan et al. 2009).

The significant alteration of sphingolipids in the midbrain could explain the locomotion and exploratory behaviour deficits of warfarin treated rats when they were subjected to the open field test. The mesencephalic locomotor region is located at the junction between the midbrain and hindbrain and is closely associated with controlling

motor movements. Mesencephalic locomotor region stimulation induces locomotion with an intensity that increases with the stimulation strength (Le Ray, Juvin et al. 2011). Also, the substantia nigra portion of the midbrain plays a critical role in the motor system of the basal ganglia pathway (Yang, An et al. 2011). Animal studies show that the ventral tegmental area of the midbrain is associated with movement and exploratory behaviours. The microinjections of dopaminergic or ovarian hormones- 17α estradiol or progesterone- into the ventral tegmental area increase the locomotor activity and exploratory behaviour in rats (Frye and Rhodes 2008). It has also been reported that in patients with Parkinson's disease, midbrain dopaminergic neurons regulate movement and emotion; their degeneration can be linked to motor and cognitive impairment (Heyer, Pani et al. 2012).

Warfarin administration was also associated with higher levels of total gangliosides in the frontal cortex, mainly explained by higher levels of GT1b. We also observed a higher level of GT1b in the striatum, and a significantly lower concentration of GD1a in the hippocampus.

Gangliosides interact with numerous regulatory proteins in the nervous system (Furukawa, Ohmi et al. 2011; Yu, Tsai et al. 2012). During brain development, they participate in many important functions such as survival, proliferation, and differentiation (Yu, Nakatani et al. 2009). Also, they act as regulator factors in the maintenance and repairing of nerve tissues (Kittaka, Itoh et al. 2008; Ohmi, Tajima et al. 2009).

Given the biological role of GT1b as a cellular apoptotic and neurotoxic agent towards dopaminergic neurons and GD1a as a modulatory survival and proliferation factor of neuronal cells (described in section 3-2-3), our results suggest that a high level of GT1b in the striatum and frontal cortex and low level of GD1a in the hippocampus might contribute to the poor performance of warfarin-treated rats in the Morris water maze test due to the link of these regions to the cognitive process. The hippocampus is the most important brain region with respect to learning and the consolidation of information from short-term to long-term memory and spatial navigation. It also plays a critical role in the Morris water maze paradigm (Redish and Touretzky 1998). The frontal cortex is also important as it contributes to executive and cognitive functions, planning of movement,

recent memory and some aspects of emotion (Chudasama 2011). Finally, the striatum plays a role in the refinement and control of motor movement that could also address the motor impairment of rats in the open field test (Balleine, Delgado et al. 2007; Haber 2011).

Finally, warfarin treatment resulted in a loss of the MK-4-sphingolipids correlation. In control Wistar rats, MK-4 concentration was positively correlated with sphingomyelin, sulfatide, and negatively correlated with gangliosides. Our findings are in line with those reported by Carrie *et al.* on Sprague Dawley rats fed the same VK-containing diet (750 $\mu\text{g}/\text{Kg}$ diet) (Carrie, Portoukalian et al. 2004). In contrast, no correlation was observed between MK-4 and any class of sphingolipids in warfarin-treated rats, suggesting a loss of the MK-4 and sphingolipids correlation as a result of warfarin treatment.

Whether warfarin treatment represents a risk factor for cognitive function in the clinical setting needs to be assessed prospectively. Cognitive impairment has been reported in subjects who suffer from various cardiovascular disorders, so patients undergoing warfarin therapy are clearly at risk of cognitive dysfunction (Alwerdt, Edwards et al. 2013). Given this confounding factor, we therefore need to be cautious in extrapolating results of the present study to the clinical setting. Additionally, the dosage of warfarin administered to rats in the present study was much higher compared to that given to patients with heart disease. To address this, studies could be conducted whereby cognitive function is investigated during different treatment periods or different warfarin dosage regimes. For instance, the cognitive functions of subjects who have undergone hip surgery and who are generally prescribed short- term warfarin therapy could be compared with those of patients undergoing long- term treatment for cardiovascular disease. Similarly, animal studies using different doses of warfarin could be used to better understand the effect of warfarin on cognitive function and behavior.

Moreover the intracellular signalling pathway of Gas 6 expression, as the main VKDP involved in cell survival, cell growth, and myelination of the central nervous system needs to be assessed to better determine the detrimental effects of warfarin in relation to cognition.

In conclusion, this study has shown that warfarin-induced VK deficiency resulted in a deficit in learning ability and lowered exploratory locomotor activity in rats, a finding associated with a dramatic decrease of MK-4 and an alteration of the sphingolipids profile in the brain.

Conclusion

We found that warfarin treatment in rats is associated with a dramatic decrease in MK-4- the main cerebral K vitamer - in all the regions of the brain. Also, warfarin altered the sphingolipid profile especially in the midbrain and frontal cortex, resulting in a loss of correlation with tissue MK-4. Moreover, warfarin administration was linked to a significant elevation of cytotoxic GT1b in the frontal cortex and striatum and a significant decline in cytoprotective GD1a in the hippocampus. These findings were associated with cognitive impairment, hypoactivity and lower exploratory behaviour in warfarin-treated rats. These results point to the potential detrimental effect of warfarin compounds on brain functions. Findings from the present study are important in light of the large number of individuals with thromboembolic conditions treated with coumarinic derivatives. Additional studies are needed to determine whether warfarin doses used in the clinical settings pose a cognitive risk for patients treated with this drug.

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