Original Paper

Regulation of Neuronal Stem Cell Proliferation in the Hippocampus by Endothelial Ceramide

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Key Words

Neuronal stem cells • Endothelial cells • Acid sphingomyelinase • Ceramide • Proliferation

Abstract

Background/Aims: Major depressive disorder is one of the most common diseases in western countries. The disease is mainly defined by its psychiatric symptoms. However, the disease has also many symptoms outside the central nervous system, in particular cardiovascular symptoms. Recent studies demonstrated that the acid sphingomyelinase/ceramide system plays an important role in the development of major depressive disorder and functions as a target of antidepressants. **Methods:** Here, we investigated (i) whether ceramide accumulates in endothelial cells in the neurogenic zone of the hippocampus after glucocorticosterone-mediated stress, (ii) whether ceramide is released into the extracellular space of the hippocampus and (iii) whether extracellular ceramide inhibits neuronal proliferation. Ceramide was determined in endothelial cell culture supernatants or extracellular hippocampus extracts by a kinase assay. Endothelial ceramide in the hippocampus was analyzed by confocal microscopy of brain sections stained with Cy3-labelled anti-ceramide antibodies and FITC-Isolectin B4. Neuronal proliferation was measured by incubation of pheochromocytoma neuronal cells with culture supernatants and extracellular hippocampus extracts. **Results:** Treatment of cultured endothelial cells with glucocorticosterone induces a release of ceramide into the supernatant. Likewise, treatment of mice with glucocorticosterone triggers a release of ceramide into the extracellular space of the hippocampus. The release of ceramide is inhibited by concomitant treatment with the antidepressant amitriptyline, which also inhibits the activity of the acid sphingomyelinase. Studies employing confocal microscopy revealed that ceramide is formed and accumulates exclusively in endothelial cells in the hippocampus of stressed mice, a process that was again prevented by co-application of amitriptyline. Ceramide released in the culture supernatant or into the extracellular space of the hippocampus reduced proliferation of neurons *in vitro*. **Conclusion:** The data suggest a novel model for the pathogenesis of major depressive disorder, i.e. the release of ceramide-enriched microvesicles from endothelial

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cells that negatively affect neuronal proliferation in the hippocampus, but may also induce cardiovascular disease and other systemic symptoms of patients with major depressive disorder.

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Introduction

Major depressive disorder, also named endogenous depression, is one of the most common diseases with a lifetime prevalence of more than 10% [1, 2]. Approximately 10% of patients with severe major depressive disorder attempt suicide resulting in a significant mortality of the disease [1, 3]. It is therefore surprising that the molecular mechanisms that lead to such a severe, common and often chronic disease are largely unknown. The disease is usually defined by its psychiatric symptoms such as depressed mood, melancholia, inability to feel, loss of interest, anhedonia, fear, feelings of worthlessness, weight loss, insomnia, and concentration deficits [1-3]. However, most patients also suffer from symptoms that affect the cardiovascular system such as high blood pressure, heart disease and arteriosclerosis, but also osteoporosis, loss of weight and metabolic changes such as adrenocortical activation, increased plasma concentrations of proinflammatory cytokines, low serum HDL cholesterol concentrations and a dysfunction of the hypothalamic-pituitary-adrenal axis [4-11].

It is unclear how these systemic changes are induced by isolated alterations of the central nervous systems and it might be more appropriate to envision major depressive disorder as a systemic disease [12].

Many antidepressants inhibit the uptake of monoaminergic transmitters and based on this finding it was postulated that major depressive disorder is caused by low concentrations of monoaminergic transmitters in the synaptic space. However, this monoaminergic hypothesis of major depression fails to explain the delayed onset of the therapeutic effects of antidepressants, which usually reduce the symptoms after 2-4 weeks of treatment, since the increase of neurotransmitters in the synaptic space is rapidly induced by these drugs [13]. Further, some antidepressants, for instance tianeptine, even promote neurotransmitter uptake [14]. Therefore novel concepts to explain major depressive disorder were developed and recent concepts focus on a reduced neurogenesis in the hippocampus as one of the major pathogenetic mechanisms for major depressive disorder [15-19]. The human brain produces approximately 700 neurons daily, mainly in the hippocampus and the prefrontal cortex [16, 17]. It is assumed that major depressive disorder is caused, at least in part, by a reduction of neurogenesis in the hippocampus. This is consistent with clinical findings demonstrating a size-reduction of the hippocampus in patients with major depression and animal models showing a reduced neurogenesis in stress-induced major depression [20-22]. The reduced neurogenesis in the hippocampus in patients with major depression might result in a rarefaction of neuronal networks and finally the functional failure of these networks with the consequence of major depression [3, 12, 18]. Antidepressants increase neurogenesis and neuronal maturation thereby restoring these networks [18, 19, 23], which requires 2-4 weeks, a time frame that is in accordance with the delayed actions of antidepressants. On a molecular basis we were recently able to show that antidepressants increase neurogenesis by an inhibition of the acid sphingomyelinase/ceramide system [12]. The acid sphingomyelinase is an ubiquitously expressed enzyme that cleaves sphingomyelin to generate ceramide [24, 25]. Ceramide inhibits proliferation in most cells or even induces cell death [26]. The inhibition of the acid sphingomyelinase by antidepressants results in a reduction of ceramide and thereby in an increase of neurogenesis in the hippocampus [12, 27].

Many patients with major depressive disorder also show increased levels of glucocorticoids in the blood, which might reduce neurogenesis [28, 29]. However, not all patients show an alteration of glucocorticoid-levels and thus a simple increase of glucocorticoids does not explain the full clinical picture of major depressive disorder [28, 29]. However, prolonged application of glucocorticoids is a good model to induce many symptoms of major depression in animal studies and is widely used as stress model [12].

The underlying pathogenesis of major depressive disorder is still unknown. It is also unknown how stress affects neuronal stem cells in the hippocampus and regulates their proliferation. Further, major depressive disorder seems to be a systemic disease that affects many organs and not just the brain or even selectively the hippocampus. Therefore, we investigated whether a stress-induced major depression alters the activity of the acid sphingomyelinase/ceramide system in endothelial cells, whether such an alteration is corrected by antidepressants and whether ceramide released by endothelial cells is able to regulate neuronal proliferation. Such a change of ceramide in endothelial cells might also explain the extraneural symptoms of major depressive disorder.

Materials and Methods

Mice

C57BL/6 wildtype mice were treated for 14 days with 250 mg/L glucocorticosterone in 0.9% NaCl used as drinking water. Glucocorticosterone was dissolved at 125 mg/mL in dimethylsulfoxide and then further diluted. Amitriptyline was applied at 120 mg/L in 0.9% NaCl as drinking water [12]. Amitriptyline application was started 2 days after initiation of the glucocorticosterone. All studies were performed in accordance with animal permissions of the Regierungspraesidium Düsseldorf and the Institutional Animal Care and Use Committee, Cincinnati.

Chemicals

All Chemicals were from Sigma-Aldrich if not otherwise noted.

Endothelial (bEnd3) cells

bEnd3 cells were obtained from ATCC and grown in RPMI-1640, 10 mM HEPES (pH 7.4, Carl Roth GmbH), 2 mM L-glutamine, 1 mM sodiumpyruvate, 100 μ M non-essential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin (all from Invitrogen) and 10 % fetal calf serum (PAA Laboratories GmbH) at 37°C and 5% CO₂. Prior to treatment with glucocorticosterone the cells were transferred to 24 well plates. After the indicated treatment, the supernatants were removed, centrifuged for 10 min at 1000xg to pellet cells, the supernatants were transferred into new tubes and shock-frozen in liquid nitrogen.

Neuronal pheochromocytoma-12 (PC-12) cells

PC-12 cells were grown in RPMI-1640, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodiumpyruvate, 100 μ M non-essential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% fetal calf serum at 37°C and 5% CO₂. To determine the effect of ceramide on the proliferation of PC-12 cells, the cells were incubated for 48 hrs with (i) 10 μ M C16 ceramide, (ii) cell culture supernatants from glucocorticosterone, glucocorticosterone + amitriptyline or amitriptyline treated or untreated endothelial cells or (iii) with extracts (see below) of the hippocampus of mice that were either left untreated or treated with amitriptyline, glucocorticosterone or glucocorticosterone + amitriptyline. To this end, the PC-12 cells were aliquoted and resuspended in the cell culture supernatants. C16 ceramide or brain extracts were directly added to the PC-12 cells in the medium above. The experiment started with 200 000 cells/mL medium. Proliferation was measured after 48 hrs incubation by counting the cell numbers in a Neubauer-chamber. In addition, cells were labelled with [³H]Thymidine during the treatment. Cells were washed after 48 hrs incubation and the cells were lysed in distilled water. The DNA was then pelleted, washed and incorporation of [³H]Thymidine was determined by liquid scintillation counted.

Hippocampus extracts

Mice were sacrificed, the brain removed, the hippocampus carefully exposed and the tissue was homogenized in 300 μ L HEPES/Saline (H/S) (132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄) in a loose Dounce homogenizer. This method left the cells intact, which was confirmed by Trypan blue staining, but resulted in complete homogenization of the tissue. Cells were pelleted by a 10 min centrifugation at 1000xg, 4°C, the supernatants were collected and shock-frozen in liquid nitrogen.

Acid sphingomyelinase activity

Supernatants were thawed and 50 μ L aliquots were added to 300 μ L of a buffer consisting of 250 mM sodiumacetate (pH 5.0) and 0.1% NP-40. The reaction was started by addition of 50 nCi [14 C]-sphingomyelin per sample (Perkin Elmer; 52 mCi/mmol). To this end, the substrate [14 C]-sphingomyelin was dried in a speedvac, resuspended in 250 mM sodiumacetate (pH 5.0) and 0.1% NP-40 and bath sonicated for 10 min to obtain micelles. The samples were incubated for 60 min at 37°C, extracted in 800 μ L $\text{CHCl}_3\text{:CH}_3\text{OH}$ (2:1, v/v), phases were separated and the radioactivity in an aliquot of the upper phase was determined by liquid scintillation counting. The activity of the acid sphingomyelinase results in the release of [14 C]-phosphorylcholine from [14 C]-sphingomyelin. Since [14 C]-phosphorylcholine is water soluble, while [14 C]-sphingomyelin is not, they can be easily separated and the amount of [14 C]-phosphorylcholine in the aqueous phase allows to calculate the enzyme activity.

Sphingosine kinase activity

To measure the activity of sphingosine kinases in the above described endothelial cell supernatants, 50 μ L of the supernatants were added to 100 μ L kinase assay buffer consisting of 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl_2 , 1 mM ATP, 10 μ Ci [32 P] γ ATP and 2 nmol sphingosine (Avanti-Polar Lipids). Samples were incubated for 60 min at 37°C, stopped by addition of 20 μ L 1N HCl and extracted in 800 μ L $\text{CHCl}_3\text{:CH}_3\text{OH}/1\text{N HCl}$ (100:200:1, v/v/v), followed by addition of each 240 μ L CHCl_3 and 2 M KCl. The lower phase was collected, dried, dissolved in 20 μ L $\text{CHCl}_3\text{:CH}_3\text{OH}$ (1:1, v/v), spotted onto Silica G60 TLC-plates and the samples were developed using $\text{CHCl}_3\text{:CH}_3\text{OH}/\text{acetic acid}/\text{H}_2\text{O}$ (90:90:15:5, v/v/v/v). TLC-plates were analyzed in a phosphoimager and the activity of sphingosine kinase was calculated by comparison of the values with a standard curve using defined amounts of sphingosine and recombinant sphingosine kinase (R&D).

Ceramide measurements

150 μ L aliquots of the cell supernatants or hippocampus extracts were added to 600 μ L $\text{CHCl}_3\text{:CH}_3\text{OH}:1\text{N HCl}$ (100:100:1, v/v/v). Phases were separated, the lower phase was collected, dried, lipids were resuspended in 20 μ L of a detergent solution consisting of 7.5% (w/v) n-octyl-glucopyranoside and 5 mM cardiolipin in 1 mM diethylenetriaminpentacetic acid (DTPA), 10 min sonicated in a bath sonicator and the kinase reaction was started by addition of 70 μ L kinase buffer consisting of 10 μ L diacylglycerol (DAG) kinase (GE Healthcare Europe, Munich, Germany), 0.1 M imidazole/HCl (pH 6.6), 0.2 mM DTPA (pH 6.6), 70 mM NaCl, 17 mM MgCl_2 , 1.4 mM ethylene glycol tetraacetic acid, 2 mM dithiothreitol, 1 μ M adenosine triphosphate (ATP), and 10 μ Ci [32 P] γ ATP. The kinase reaction was performed for 60 min at 22°C, samples were then extracted in 1 mL $\text{CHCl}_3\text{:CH}_3\text{OH}:1\text{N HCl}$ (100:100:1, v/v/v), 170 μ L buffered salt solution (135 mM NaCl, 1.5 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM Glucose, 10 mM HEPES [pH 7.2]) and 30 μ L of 100 mM EDTA. Phases were separated, the lower phase was collected, dissolved in 20 μ L $\text{CHCl}_3\text{:CH}_3\text{OH}$ (1:1, v/v) and lipids were separated on Silica G60 TLC plates using chloroform/acetone/methanol/acetic acid/ H_2O (50:20:15:10:5, v/v/v/v/v). TLC-plates were analyzed on a phosphoimager and ceramide amounts were determined by a standard curve with defined amounts of C16 und C24 ceramide as substrate.

Immunohistochemical analysis of ceramide in endothelial cells of the hippocampus

Mice were sacrificed, brains were removed and immediately shock-frozen in Tissue-Tek. Frozen sections of the brain were obtained, dried on air for 5 min, fixed in ice-cold acetone for 10 min, washed 3-times in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) and unspecific binding sites were blocked in H/S + 5% FCS. The sections were washed and incubated with anti-ceramide-antibodies (Glycobiotech; 1:100 diluted in H/S + 1% FCS) for 45 min at room temperature. The samples were then washed 3-times in PBS + 0.05% Tween 20 for each 5 min, once in PBS and stained with a Cy3-labelled anti-mouse-IgM antibody. The samples were washed again 3-times, 5 min each in PBS + 0.05% Tween 20 and once in PBS and were then incubated for 12 hrs at 4°C with FITC-labelled Isolectin B4 (1:50 in H/S + 1% FCS diluted). Samples were washed again as above, embedded in Mowiol and analyzed on a Leica TCS DMIRE.

Statistical analysis

Data are shown as mean $\pm$ SD and examined with analysis of variance (ANOVA) and post hoc tests. A *P* value of 0.05 or less (two-tailed) was considered indicative of statistical significance.

Results

To test whether endothelial cells release ceramide into the extracellular space upon treatment with glucocorticoids, we incubated bEnd3 endothelial cells with glucocorticosterone for 48 hrs and determined the concentration of ceramide in the supernatant. To this end, any remaining cells present in the supernatants were pelleted by centrifugation, the supernatants were collected and lipids were extracted from these supernatants. Ceramide was then quantified employing a ceramide-kinase assay. The studies revealed a marked release of ceramide from the glucocorticoid-stressed endothelial cells (Fig. 1). Treatment of the endothelial cells with amitriptyline, a functional blocker of the acid sphingomyelinase [27, 30, 31] and well-established antidepressant, prevented the release of ceramide induced by glucocorticoids and even reduced the baseline release of ceramide from endothelial cells (Fig. 1).

Next, we investigated whether stressed endothelial cells also release acid sphingomyelinase and/or sphingosine kinase in addition to ceramide. Release of the acid sphingomyelinase would promote stress by further generation of ceramide, while release of sphingosine kinase would counteract stress by generation of sphingosine 1-phosphate as recently shown for hepatocytes after ischemia/reperfusion injury [32]. However, we were unable to detect measurable amounts of acid sphingomyelinase or sphingosine kinase in the supernatant of glucocorticosterone-stressed endothelial cells (not shown).

To translate the *in vitro* findings into the *in vivo* situation, we tested whether we are able to detect ceramide-containing microvesicles in the hippocampus of stressed, amitriptyline-treated or unstressed mice.

To this end, we prepared the hippocampus, homogenized the tissue in a loose dounce homogenizer, pelleted cells and determined ceramide in the supernatants. Trypan Blue

Fig. 1. Cultured endothelial cells release ceramide after glucocorticoid-mediated stress. bEnd3-endothelial cells were incubated for 48 hrs with glucocorticosterone, the supernatants were removed, cells were pelleted and ceramide was determined in the supernatants. Glucocorticosterone induced a marked release of ceramide from the endothelial cells into the extracellular medium, which was blocked by co-incubation with amitriptyline, an inhibitor of the acid sphingomyelinase. Shown are the mean $\pm$ SD of each 6 independent samples. * $p < 0.05$ for significant differences compared to untreated controls and $\Delta p < 0.05$ for significant differences compared to glucocorticosterone alone, ANOVA.

Fig. 2. Ceramide is increased in extracellular extracts of the hippocampus of stressed mice. Mice were treated with glucocorticosterone, amitriptyline or glucocorticosterone + amitriptyline or left untreated. The hippocampus was removed, carefully homogenized, cells were pelleted and ceramide in the supernatants was measured by a kinase assay. The data reveal that ceramide is present in the extracellular space of the hippocampus. Extracellular ceramide is increased after treatment of the mice with glucocorticosterone. Amitriptyline prevents the effects of glucocorticosterone on the extracellular release of ceramide in the hippocampus. Shown are the mean $\pm$ SD of each 5 mice, * $p < 0.05$ compared to untreated mice, $p < 0.05$ compared to glucocorticosterone alone, ANOVA.

Fig. 3. Ceramide accumulates in endothelial cells in the neurogenic zone of the hippocampus. Untreated mice show low levels of ceramide within the endothelial cells of the neurogenic zone of the hippocampus, which is even further reduced by amitriptyline (A, C). Glucocorticosterone markedly increases the levels of ceramide within these endothelial cells, an effect that is prevented by co-treatment of the mice with amitriptyline (B, C). Brain sections were stained with FITC-conjugated Isolectin B4 that specifically binds to endothelial cells and Cy3-labelled anti-ceramide-antibodies and analyzed by confocal microscopy. Displayed are the single stainings and the overlays (right panels). Panel C shows enlarged areas of interests of the overlays in A and B. Demonstrated are representative results from each 6 mice per group. The co-localization of endothelial cells (green) and ceramide (red) in the overlays appears in yellow and is indicated by arrows.

stainings confirmed the integrity of the cells after homogenization. These experiments revealed the presence of ceramide in these hippocampus extracts (Fig. 2). Since ceramide is insoluble in water, it is most likely that ceramide is present in microvesicles. Treatment with glucocorticosterone markedly increased the concentration of ceramide/ceramide-enriched microvesicles in the hippocampus extracts, which was normalized by treatment with amitriptyline (Fig. 2). Amitriptyline reduced the concentration of ceramide in extracellular hippocampus extracts even in non-stressed mice (Fig. 2).

Fig. 4. C16 ceramide, ceramide-enriched cell culture supernatants or hippocampus extracts inhibit the proliferation of neurons. Treatment of neuronal PC-12 pheochromocytoma cells with 10 μ M C16 ceramide, supernatants (Sup.) from glucocorticosterone-treated (tr.) bEnd3-endothelial cells (A, B) or hippocampal extracts from glucocorticosterone-treated mice (C, D) inhibits proliferation of neurons. Concomitant application of amitriptyline prevents glucocorticosterone-induced release of ceramide by bEnd3 cells and within the hippocampus of cells or mice, respectively (A-D). Amitriptyline alone was without effect on proliferation (A-D). Addition of anti-ceramide-antibodies (indicated as anti-ceramide) to the hippocampus extracts from stressed mice prevented the inhibitory effect of these extracts on cell proliferation (C, D). Glucocorticosterone added to cell culture supernatants (indicated as Sup. + Corticosterone) obtained from untreated bEnd3-endothelial cells was without effect on the proliferation of PC-12 cells (A, B). Cell proliferation was measured by Trypan-Blue staining and counting cells daily in a Neubauer-chamber (A, C) and by [3 H]Thymidine-labelling of the cells followed by extraction of the DNA (B, D). Shown are the mean $\pm$ SD of each 6 cells or 5 mice, respectively, * p <0.05 compared with the untreated controls and, Δ or # p <0.05 compared with corticosterone alone, ANOVA.

To demonstrate that ceramide is increased in endothelial cells of the hippocampus after stress and normalized within these cells upon treatment with amitriptyline, we stained sections of the hippocampus with Cy3-coupled anti-ceramide-antibodies and FITC-conjugated Isolectin B4 that specifically binds to endothelial cells [33]. Confocal microscopy studies revealed that

ceramide increased in endothelial cells of the hippocampus after treatment of the mice with glucocorticosterone (Fig. 3A-C). This increase was prevented by concomitant treatment of the mice with amitriptyline (Fig. 3A-C).

These data demonstrate that ceramide is released within endothelial cells of the hippocampus upon stress and that amitriptyline normalized endothelial ceramide concentrations in stressed mice. Further, ceramide is detected in the extracellular space of the hippocampus, most likely in microparticles.

We therefore investigated whether ceramide-containing hippocampus extracts, ceramide-containing endothelial cell culture supernatants or pure C16 ceramide influence the proliferation of neuronal cells. To this end, neuronal PC-12 pheochromocytoma cells were incubated with 10 μ M ceramide, supernatants of bEnd3 endothelial cells or hippocampus extracts of mice, respectively, treated with glucocorticosterone, amitriptyline or glucocorticosterone + amitriptyline. Controls were neuronal PC-12 cells incubated with supernatants or hippocampus extracts from untreated bEnd3 endothelial cells or mice, respectively. Ceramide was neutralized by addition of anti-ceramide antibodies to the extracts. Further, we added glucocorticosterone to the cell culture supernatants from untreated endothelial cells to exclude an effect of the glucocorticosterone only.

The experiments revealed that C16 ceramide, ceramide-containing cell culture supernatants from stressed endothelial cells and microparticles from the hippocampus of glucocorticosterone-stressed mice inhibited the proliferation of neuronal PC-12 cells (Fig. 4A-D). In contrast, supernatants, from bEnd3-endothelial cells or extracts from the hippocampus of mice, respectively, that were treated with glucocorticosterone + amitriptyline did not affect proliferation of neuronal PC-12 cells (Fig. 4A-D). Supernatants or hippocampus extracts from bEnd3-endothelial cells or mice, respectively, treated with amitriptyline only, were without effect on the proliferation of PC-12 cells (Fig. 4A-D). Likewise, addition of glucocorticosterone to supernatants after their isolation from untreated bEnd3-endothelial cells did not alter proliferation of PC-12 cells (Fig. 4A-D), excluding that glucocorticosterone by itself mediated the effects on proliferation. Neutralization of ceramide in the hippocampus extracts from stressed mice by addition of anti-ceramide antibodies prevented the growth inhibitory effect on PC-12 cells of these extracts.

Discussion

Here we provide a novel concept for the genesis of central nervous and systemic, extraneural symptoms on patients with major depressive disorder: We demonstrate that glucocorticosterone-stressed endothelial cells release ceramide, which is prevented by amitriptyline. Most importantly, endothelial cells of small vessels in the neurogenetic zone of the hippocampus accumulate ceramide upon stressing the mice with glucocorticosterone, an alteration that is also prevented by co-treatment of the mice with amitriptyline, a well-known and often-used antidepressant and functional inhibitor of the acid sphingomyelinase [27]. The accumulation of endothelial ceramide correlates with a release of ceramide into the extracellular space of the hippocampus in stressed mice, which is reduced to basal levels by treatment with amitriptyline. Both, the supernatants of stressed endothelial cells as well as the extracts from the hippocampus of stressed mice reduced neuronal proliferation, which was restored by treatment of the endothelial cells or the mice, respectively, with amitriptyline or by neutralization of ceramide within the extracts using anti-ceramide antibodies.

Our data show that ceramide in the supernatant of endothelial cells or the extracellular space of the hippocampus regulates proliferation of neuronal cells. Antibodies neutralizing ceramide prevent this effect, which is in turn mimicked by C16-ceramide, a natural ceramide that is often involved in the mediation of stress [3] and that has been shown to reduce neuronal proliferation after direct injection into the hippocampus [12]. Since ceramide is insoluble in water it can only exist in the aqueous extracellular phase as a part of exosomes, microparticles or in micelles. It is beyond the focus of the present manuscript to exactly define

the nature of the vesicles that contain ceramide released by endothelial cells upon stress. Therefore, we use the terminology microvesicles. Microvesicles are often characterized by their source and size: Microparticles are released from the plasma membrane, a process that requires activity of the acid sphingomyelinase [34]. They are usually pelleted at 25,000xg. Exosomes are smaller than microparticles and pellet at 100,000xg. They are released upon fusion of multivesicular bodies with the plasma membrane, requiring activity of the neutral sphingomyelinase [35]. Microparticles were recently implied in the release of inflammatory mediators by glia cells [34] and also in ischemic damage of the brain [36] as well as Alzheimer degeneration [37]. However, a role of endothelial-derived extracellular ceramide in regulation of neuronal cell proliferation has not previously been shown.

It will be very interesting to determine the enzymes and mechanisms involved in the formation of ceramide containing vesicles as well as the exact nature of these vesicles released from endothelial cells upon stress. At present, the regulation of the release of microvesicles from endothelial cells by glucocorticoids is poorly characterized: A study by Thai et al. [38] demonstrated that dexamethasone triggers the release of annexin A1-containing microparticles from endothelial cells, while Zhu et al. [39] reported that dexamethasone inhibits the constitutive release of microparticles from human umbilical vein endothelial cells. We assume that different endothelial cell types show a different response to glucocorticoids, since the function of umbilical vein endothelial cells, a fenestrated renal endothelial cell and a very tight endothelial cell in the stem cell niche of the hippocampus, to name a few examples, have very different functions. Moreover, the effect of glucocorticoids on ceramide in microvesicles released by endothelial cells has not been investigated so far.

The present studies demonstrate a specific accumulation of ceramide in endothelial cells in the hippocampus and a release of ceramide into the extracellular space of the hippocampus upon application of stress and a correction of these stress effects by amitriptyline. Major depressive disorder is not only a disease of the brain with psychiatric symptoms, but also a systemic disease with cardiovascular symptoms or osteoporosis [1-11]. These symptoms cannot be explained by an isolated alteration of the hippocampal neurogenesis or a reduced concentration of neurotransmitters in the synaptic space. Here, we suggest a novel model to explain the various symptoms of major depressive disorder: The accumulation of ceramide within endothelial cells of the microcirculation results in the extracellular release of ceramide, in particular in ceramide-enriched microvesicles, that reduces proliferation of neuronal stem cells being in close vicinity to endothelial cells in the vascular-stem cell niche of the hippocampus. Endothelial ceramide has been shown to be critically involved in arteriosclerosis and heart disease [40-43], in accordance with the present concept. The present concept is also in accordance with the finding that direct injection of micellar ceramide into the hippocampus induces major depressive disorder symptoms in mice [12]. In addition, application of decanoylamino-3-morpholino-1-propanol (PDMP), which prevents glycosylation of ceramide and results in a cellular accumulation of ceramide induces major depressive-like symptoms in mice [12]. PDMP is, however, unable to cross the blood brain barrier, and therefore is only able to directly act on endothelial cells, but not on neuronal stem cells [44]. Finally, several studies demonstrated that Vascular Endothelial Growth Factor (VEGF), which promotes endothelial cell proliferation and stabilizes endothelial cells [45, 46], acts anti-depressive. Since VEGF inhibits the acid sphingomyelinase [47], it is possible that this anti-depressive effect of VEGF is related to inhibition of the acid sphingomyelinase/ceramide system in endothelial cells. However, neuronal stem cells express VEGF-receptor 2 [48] and it is therefore also possible that VEGF does not only act on endothelial cells but also directly on neuronal stem cells after crossing the blood-brain barrier.

It is interesting to speculate how the acid sphingomyelinase in endothelial could be manipulated in addition to treatment with antidepressants or VEGF. It might be possible to change endothelial sphingolipids by nutrition [49], although it is not known whether these effects would be strong enough to reduce ceramide formation. It might be also possible that polymorphisms and the sex influence the activity of the acid sphingomyelinase in and the release of ceramide from endothelial cells [50, 51], in particular since major depression

seems to be more common in women. However, the role of these factors for the release of ceramide from endothelial cells must be carefully explored and is beyond the focus of the present manuscript.

Extracellular ceramide may act on cells by changing the biophysical properties of biomembranes [52]. Ceramide integrates into membranes and forms large ceramide-enriched membrane domains that are very hydrophobic, relatively rigid and almost in a gel-like state [52, 53]. These ceramide-enriched membrane platforms are able to trap and cluster stress receptors and associated signalling molecules, while other signalling molecules are excluded from these domains [26, 52, 53]. The clustering of stress receptors in ceramide-enriched membrane domains might result in their activation and thereby in inhibition of cell proliferation and/or even cell death [26, 52, 53].

Previous data from our laboratory demonstrated that glucocorticosterone does not alter overall ceramide levels in the hippocampus and, moreover, also reduces neurogenesis and induces major depression in acid sphingomyelinase-deficient mice [12]. Acid sphingomyelinase-deficient mice do not respond to tricyclic antidepressants anymore [12] indicating that inhibition of the acid sphingomyelinase is required for the effects of these antidepressants. On the other hand, neurogenesis was reduced and major depression induced in acid sphingomyelinase-transgenic mice and acid ceramidase-heterozygous mice showing an increase of ceramide in the hippocampus due to the overproduction of ceramide or the reduced consumption of ceramide, respectively [12]. Direct injection of ceramide into the hippocampus had the same effects [12]. These data show that ceramide itself reduces neurogenesis and induces major depression symptoms. However, glucocorticosterone does not act via an activation of the acid sphingomyelinase, since it also induces major depression in acid sphingomyelinase-deficient mice [12]. Thus, glucocorticosterone could increase ceramide levels in endothelial cells observed in the present study by an activation of the neutral sphingomyelinase, the ceramide synthesis pathway or by an inhibition of ceramide metabolism/consumption. The amount of ceramide specifically produced and released in endothelial cells is too low to be detected in lysates of the whole hippocampus, while the local concentration of ceramide within the vascular niche of stem cells might be high enough to prevent stem cell proliferation [12]. Antidepressants inhibit the acid sphingomyelinase and act by reducing the total concentration of ceramide within endothelial cells and thereby also reduce the amount of ceramide released into the extracellular space of the hippocampus. The reduction of total ceramide by antidepressants counteracts the effects of glucocorticosterone on endothelial cells and thereby restores neurogenesis and prevents major depression. If this model is correct, amitriptyline should also prevent extraneural symptoms of major depressive disorder, for instance cardiovascular disease or osteoporosis.

In summary, our data show that stressed endothelial cells release ceramide into the extracellular space upon application of glucocorticosterone. Amitriptyline, an inhibitor of the acid sphingomyelinase and a commonly used antidepressant, prevents the release of ceramide. Ceramide inhibits proliferation of neuronal stem cells. These data suggest a novel model for the pathogenesis of major depressive disorder: Ceramide levels in endothelial cells of the neurogenic zone regulate neurogenesis in the hippocampus. This model may also explain the systemic symptoms of many patients with major depressive disorder.

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Disclosure Statement

The authors have no conflicts to declare.

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