The effect of prolonged simvastatin application on serotonin uptake, membrane microviscosity and behavioral changes in the animal model

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HIGHLIGHTS
• Prolonged simvastatin treatment causes decrease of membrane microviscosity.
• Prolonged simvastatin treatment causes decrease of serotonin transporter activity.
• Behavioral changes after simvastatin treatment — anti-depressant effect?

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ABSTRACT
Simvastatin and other statins (HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors) are extensively used in clinical practices and are very effective in decreasing serum low-density lipoprotein cholesterol. However, their effect on cholesterol synthesis in central nervous system and its behavioral consequences have not been fully understood yet. We have studied selected biologic traits potentially affected by statin treatment — serotonin (5-HT) uptake in platelets, membrane microviscosity in erythrocytes, cholesterol level in the brain (amygdala; hippocampus and prefrontal cortex), as well as behavioral changes in an elevated plus maze and open field test in male Long-Evans rats, which were treated by simvastatin (30 mg/kg per day) for 2 or 4 weeks.

We demonstrated: 1) a decrease in both serotonin transporter (SERT) activity and membrane microviscosity after treatment with simvastatin, 2) lower cholesterol content in all tested brain regions in animals from the simvastatin treated group, and 3) longer time spent in the open arms and a higher number of entrances to the closed arms in the elevated plus maze by animals from the simvastatin group compared to animals from the control group, but no differences in behavior in the open field test.

Taken together, our results confirmed complex alterations, including behavioral changes, after the cholesterol lowering treatment. Furthermore, we discuss the possibility that the behavioral changes, traditionally interpreted as an anxiolytic effect, may be interpreted as increased impulsivity. We also confirmed that such behavioral changes may be attributed to changes in serotonergic neurotransmission.

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Abbreviations: 5-HT, Serotonin; APCI, atmosphere pressure chemical ionization; CID, collision induced dissociated; CNS, central nervous system; DPH, 1,6-Diphenyl-1,3,5-hexatriene; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EPM, elevated plus maze; FDA, Food and Drug Administration; HMG-CoA, 3-Hydroxy-3-methylglutaryl coenzyme A; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LOD, low limit of detection; LOQ, low limit of quantification; MS, mass spectrometry; OF, open field; PRP, platelet rich plasma; RE, relative error; RSD, reflex sympathetic dystrophy; SERT, serotonin transporter; TMA-DPH, 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate.

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1. Introduction

Simvastatin and other statins (HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors) have been demonstrated to reduce mortality and the risk of major cardiovascular events. However, in 2012, the U.S. Food and Drug Administration (FDA) approved a safety warning on the cognitive side effects associated with statins [1]. Psychological adverse events may include behavioral alterations such as irritability, impulsiveness and hostility [2-7], depression [8], as well as cognitive and memory impairments [9-12].

Nevertheless, the mechanisms of neuropsychiatric adverse effects have not been fully understood yet. A number of studies have reported an increased risk of violent deaths and depression in subjects with reduced serum cholesterol concentrations [13]. This can be explained by the theory proposed by Engelberg [14], and modified by Vevera et al. [13], who speculate that serotonin neurotransmission is affected via the altered microviscosity of plasma membranes and by the direct effect of cholesterol on serotonin transporter (SERT) activity caused by the lowered availability of cholesterol. Serotonin pathways function as a behavioral restraint system that inhibits impulsive behavior[15]. Reduced cholesterol levels could thus facilitate such complex behavior as violence towards the self or others.

Cholesterol is a major constituent of the human brain, and the brain is the cholesterol-richest organ containing about 20% of the body’s total cholesterol [16]. Unlike cholesterol in other organs in the periphery, brain cholesterol is primarily derived by de novo synthesis. The rate-limiting enzyme in this process is HMG-CoA reductase. The effects of HMG-CoA reductase inhibitors (statins) on cholesterol synthesis in central nervous system (CNS) and its behavioral consequences have not been fully described [17,18].

Current understanding of the impact of statins on CNS is limited due to a lack of biological mechanism-based studies, as well as the assumption that all statins have the same pharmacological effect. Nevertheless, brain cells appear to express the same control mechanisms seen in cells of other tissues for maintaining the concentration and turnover of cholesterol in the metabolically active pool and plasma membrane [17,18]. In contrast, there is a marked difference in the half-life of cholesterol i.e. a few days in plasma, whereas brain cholesterol has an extremely long half-life. In the adult human brain the estimated half-life of the bulk of cholesterol is 5 years, and it is between 2 and 6 months in the adult rat’s brain [19,20]. A major route of cholesterol turnover in the brain is its conversion to 24S-hydroxycholesterol by cholesterol 24-hydroxylase (CYP46A1), which is expressed primarily in neurons of CNS. In contrast to cholesterol, 24S-hydroxycholesterol can readily traverse the blood–brain barrier to enter the systemic circulation.

An increase in the turnover of cholesterol to its metabolite 24S-hydroxycholesterol in the prefrontal cortex of suicide cases has also recently been reported [21]. Post-mortem studies reported a significant decrease in total cholesterol levels in violent compared to non-violent suicides in the frontal cortex [22]. Another group also reported a significant decrease in cholesterol in the visual association cortex in subjects with major depressive disorder [23]. These findings together with the association of low cholesterol levels with impulsivity and depression [3] suggest a basis for the potential role of cholesterol within the CNS as a factor in the underlying neuropsychiatric pathology [21].

Brain cholesterol synthesis is affected by cholesterol-lowering medication [24,25] and it has been demonstrated that treatment with simvastatin decreases the level of cholesterol in synaptosomal membranes, affects trans-bilayer cholesterol distribution [26] and reduces cholesterol turnover in the brain [27]. On the other hand, several studies found no changes in the cholesterol content in the brain of rodents after simvastatin treatment [28-30].

The serotonin transporter (SERT) plays a crucial role in the maintenance of normal serotonergic neurotransmission. SERT controls the concentration of extracellular serotonin in the brain. An important mechanism for the modulation of SERT activity is the density of the transporter molecules at the cell membrane and their affinity to serotonin (5-HT). Previous findings indicated that SERT activity is regulated both by membrane cholesterol [31] and several regulatory proteins [32]. Platelets share some metabolic pathways with neurons, e.g. neurotransmitter metabolism and transport, and platelets can serve as models for some neuronal receptors [33-35]. Moreover, platelets are a powerful biological model to study the effects of drugs on brain cells. It is assumed that the kinetic parameters of serotonin uptake in platelets from peripheral blood can be used to characterize SERT activity in the brain [36].

The contribution of the serotonergic system to the behavioral effects of statins has also been demonstrated in preclinical models of depression-like behavior. The link between the serotonergic system and statins is supported by pharmacological studies using co-applications of statins and antidepressants. Lin et al. [37] demonstrated that simvastatin treatment facilitates the anti-depressant effect of imipramine in the chronic mild stress model in laboratory rats. Analogously, in the forced swimming test, lovastatin augments the antidepressant like effect of fluoxetine, a selective serotonin reuptake inhibitor [38]. Antidepressant facilitation can even be abolished by induced serotonin depletion [39].

Our previous research has also demonstrated changes in serotonin transmission evoked by simvastatin therapy in a clinical cholesterol-lowering trial [13]. SERT activity was significantly increased following one month of simvastatin administration; the tendency to lower the initial increase in SERT activity was evident following two months of therapy. Both membrane cholesterol and SERT activity returned to pretreatment levels after more than one year of therapy. We have hypothesized that there is a time window when patients can be vulnerable to neuropsychiatric adverse events; this window begins after one month of cholesterol-lowering therapy [13]. Conversely, analysis of case reports revealed that the median onset time for psychiatric adverse events was 41 days from the first statin administration [40].

The aim of our current study was to conduct a multi-disciplinary and comprehensive study on the effects of short- and long-term application of simvastatin on cholesterol levels in the brain, serotonin uptake, membrane microviscosity and behavior in the animal model.

2. Materials and methods

2.1. Experimental animals and simvastatin administration

The animals (n = 12) used in the experiments were adult naive male Long-Evans rats, which were 3-months-old at the beginning of the experiment. The rats were obtained from the breeding colony of the Institute of Physiology, Czech Academy of Sciences and housed in groups of three per cage (25 × 25 × 50 cm) in a temperature-controlled room (21 °C) with a 12-h light-dark cycle (the lights were on at 7:00 a.m.). Water was freely available but access to food (Altromin diet 1314) was restricted to maintain the rats at 90% of their free feeding weight (380-450 g).

Simvastatin (Simvastatin, Mylan) was administered to rats per orally. To achieve voluntary consumption of the simvastatin it was dissolved into sweetened jelly and was administered to the rats according to the procedure described in Zhang [41] at a dose of approximately 30 mg/kg per day for four weeks. We used a sucrose-derived non-caloric sweetener (Sacharin) to avoid introducing additional calories into the jelly. Three mg of simvastatin was used per gram of jelly. The amount of jelly was calculated individually according to animal’s weight. A rat weighing 400 g thus obtained approximately 4 g of jelly per day. Because the rats show innate avoidance to novel food, they were habituated to eating the jelly two days before the experiment. During this period, food-restricted animals obtained simvastatin-free jelly. The weight of the rats was regularly monitored and the dosages were adjusted continuously according to the actual weight of the rat so that the dose per kilogram per day remained constant. The animals
were randomly assigned to the group receiving a vehicle jelly with simvastatin and the vehicle jelly alone (control). Blood samples were collected at days 1, 14 and 28.

All of the procedures complied with the Animal Protection Code of the Czech Republic and with the European Council directives (2010/63/EC; 86/609/EEC). The study protocol was approved by the Animal Care Committee of the Institute of Physiology of Academy of Sciences of the Czech Republic.

2.2. Sample analysis

At the beginning of the study, as well as at day 14 of the treatment with simvastatin, peripheral blood samples (0.5 ml) were obtained from the rat tails from both the experimental and control groups. At the end of the study (at day 28 of the treatment with simvastatin), the animals were sacrificed by decapitation. Non-coagulable blood samples were obtained using K$_2$EDTA as an anti-coagulant.

Platelet-rich plasma (PRP) was separated by centrifugation at 200 × g for 30 min at 25 °C. Platelets were counted by microscopy using a counting chamber, diluted by the KH medium to a concentration of 2.10$^8$ platelet/ml and immediately used to measure the SERT activity (serotonin uptake).

The erythrocyte ghosts were prepared according to [42]. Erythrocytes were washed 3 times with an isotonic buffer. One ml of washed erythrocytes was hemolyzed in 30 ml of hypotonic buffer with 0.5 mM EGTA under vigorous agitation. The suspension was then centrifuged at 20 000 g for 20 min at 4 °C. The supernatant was discarded and the erythrocyte membranes were washed once again with a hypotonic buffer with EGTA, two times with a hypotonic buffer without EGTA and two times with an isotonic buffer. The final sediment was resuspended in a buffer volume corresponding to the initial blood volume. The membrane protein concentration was measured by the Lowry method [43]. Erythrocyte membranes were stored at −70 °C until the membrane fluidity measurements.

2.3. Serotonin uptake assessment

The kinetic parameters of the uptake of serotonin into platelets were measured using tritium labeled 5-HT [44,45]. The previously described experimental protocol was adapted [46]. Briefly, 0.1 ml of the sample (2 × 10$^7$ platelets) was mixed with the KH medium solution and incubated at 37 °C for 12 min. The measurements of platelet 5-HT uptake kinetics were initiated by adding 8 different [3H]5-HT volumes of 8 μM [3H]5-HT, so that the resultant concentrations of [3H]5-HT for platelet uptake were in the range of 10−600 nM. The final volume of the testing sample was 2 ml with a platelet concentration of 10$^7$ per ml. After adding the labeled 5-HT, the test tubes were incubated at 37 °C for 5 min. Free [3H]5-HT was then removed by rapid filtration followed by washing of the filters. The non-specific uptake and the non-specific binding of [3H]5-HT to the platelet surface or to the filter were determined from 2 °C cold samples. Tritium radiation of samples was measured on a liquid scintillation counter (LS6000IC, Beckman Instruments, Inc., Fullerton, CA, USA).

2.4. Membrane microviscosity assay

Erythrocyte membranes (red blood cell ghosts) have become the standard model for the investigation of the plasma membrane properties, including membrane fluidity [47]. Hydrophobic membrane fluorescent probes are used to determine changes in membrane microviscosity [48] based on the measurement of fluorescence anisotropy (r), which reflects the extent of probe movement restriction in an anisotropic membrane environment.

The membrane microviscosity of erythrocyte ghosts was measured using the fluorescent probes method [48] by depolarization of the fluorescence emission of 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) fluorescent probe. Dilution of the probe by up to 3 μM was performed in PBS using a Hamilton syringe immediately before measurement. PBS with a probe, PBS, and the erythrocyte ghost were mixed so that the final concentrations were 200 μg/ml of membrane protein and 2 μM of DPH or TMA-DPH. It was verified that there was no significant depolarization of fluorescence at 450 nm due to the turbidity of the ghost suspension.

Fluorescence anisotropy (r), which is in direct relation to membrane microviscosity, was measured on a FluoroMax-3 spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) at 37 °C after 60 min equilibration of the ghosts with the probe. The excitation and emission wavelengths were 350 and 450 nm, respectively, with a 2 nm bandwidth in excitation and 5 nm bandwidth in emission for both DPH and TMA-DPH. Measurements of DPH and TMA-DPH fluorescence were collected with different configurations of excitation and emission polarizers in the vertical and horizontal positions.

2.5. Chemicals and solutions

The Krebs–Henseleit medium (KH medium), which was used for the dilution of intact platelets in platelet-rich plasma, consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$·7H$_2$O, 25 mM NaHCO$_3$, and 11.1 mM glucose (pH 7.4). The KH medium was saturated with oxygen before use by percolation with a 95% O$_2$ and 5% CO$_2$ gas mixture. Eight μM of tritium-labeled 5-HT ([3H]5-HT) solution with a specific activity of approximately 25 kBq/ml was prepared by mixing the KH medium with 1 μM of unlabeled (cold) 5-HT (5-hydroxytryptamine creatinine sulphate, Sigma) and the [3H]5-HT stock solution (5-hydroxytryptamine [1H] trifluoroacetate; specific activity of 2.96 TBq/mmol, radiochemical purity >99%, American Radiolabeled Chemicals, Inc., 101 ARC Drive, St. Louis, MO 63146).

An isotonic buffer (120 mM NaCl, 10 mM KCl, 30 mM Tris, pH 7.4) and a hypotonic buffer (20-times diluted isotonic buffer, pH 7.4) were used for erythrocyte ghost preparation.

Fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma-Aldrich, St. Louis, MO, USA) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) (Molecular Probes, Eugene, Oregon, USA) were dissolved in acetone (6 mM DPH) and methanol (3 mM TMA-DPH), respectively and stored in a freezer. A phosphate buffered saline (PBS), consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$ (pH 7.4), was used during fluorescence anisotropy measurements.

2.6. Brain level of cholesterol

2.6.1. Tissue sampling

The rats were sacrificed the day after behavioral testing by decapitation and their brains were immediately removed. The hippocampus, striatum and frontal cortex were separated and immediately frozen for subsequent analysis.

2.6.2. Analytical methods for determination of cholesterol in the brain

The method consisted of a pre-treatment step, separation of cholesterol (extraction), and a detection step using liquid chromatography combined with electrospray ionization tandem mass spectrometry (HPLC–APCI-MS/MS). The pre-treatment step was performed according to the following procedure: the internal standard of cholesterol-d$_{4}$ (1 ng/10 mg of brain tissue), a mixture of solutions for extracting the contained methanol (0.300 ml/100 mg of brain tissue), and chloroform (0.6 ml/100 mg of the tissue) were added to the tissue parts of the brain samples. The mixture and the brain tissue were homogenized (ULTRATURRAX T 10 basic) and subsequently sonicated for 10 min at 4 °C. Each sample was then centrifuged at 9000 g at the temperature of 4 °C, the supernatant was separated and filtered on a 0.2 μm PTFE microfilter.
The supernatant was stripped off by nitrogen to dry. After the drying was completed, the rest was dissolved by ethanol (1 ml) and the sample was immediately analyzed by HPLC–APCI-MS/MS. The LC–APCI-MS/MS system consisted of an Accela 1250 LC chromatogram (Thermo Scientific, USA), an Accela autosampler (Thermo Scientific, USA) and a TSQ Vantage mass spectrometer (Thermo Scientific, USA). The analytes were separated on Kinetex C18 50 × 2.1 mm, 2.6 μm (Phenomenex, USA) and a mobile phase (solvent A: methanol; solvent B: 0.1% formic acid in water) in a gradient elution at the flow rate of 150 μl/min. The HPLC elution program was as follows: 20% B (1 min) → 5% B (a linear increase in 4 min) → 5% B (10 min) → 20% B (a linear decrease in 1 min) → 5% B (5 min). The column temperature was maintained at 25 °C. The sample temperature was maintained at 4 °C. The injection volume was 10 μl. Cholesterol was eluted in the retention time of 12.7 min. A mass spectrometer equipped with an atmosphere pressure chemical ionization (APCI) was used for the detection of cholesterol and their deuterium labeled internal standards (cholesterol-d6) in positive mode ionization (APCI+). The selective reaction monitoring (SRM) mode was used. The scan monitoring reactions (precursor ion → fragment ion) used for the analyses and their collision induced dissociated (CID) energy were as follows: m/z = 369.4 (corresponding to [M+H]+ dehydration ion) → m/z = 147.1 (CID = 22 eV) for cholesterol, and m/z = 375.3 → m/z = 153.1 (CID = 22 eV) for cholesterol-d6. The fragmentation ion for cholesterol and its internal standard was selected on the basis of a collision spectrum (see Fig. 1). The mass spectrometer conditions were optimized as follows: discharge current 5.0 μA, temperature of ion transfer tube 350 °C, pressure of sheath gas (nitrogen) 30 psi, and flow of auxiliary gas (nitrogen) 15 arbitrary units. The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific, USA).

2.7. Apparatuses and behavioral procedures

Behavioral testing was carried out at day 27 of simvastatin treatment.

2.7.1. Elevated plus maze

The apparatus consisted of a cross with two opposite open arms (45 cm × 10 cm) which crossed at right angles with two arms of the same size enclosed by walls 40 cm high (closed arms). All of the arms were accessible from the central platform where the arms were crossed. The whole apparatus was painted black and elevated 50 cm above the floor.

At the beginning of each session, the rat was placed on the central platform facing the closed arm. Behavior was recorded over a 10-min test session by a video recorder placed above the maze and tracked by software (Tracker, Biosignal Group US). The number of open and closed arm entries and the time spent in the particular arms was recorded and compared. During a standard elevated plus maze (EPM) test, animals are considered to be more anxious if they spend a greater proportion of time in the closed, compared to the open arms.

2.7.2. Open field test

The animals were placed individually into an open field arena (68 cm × 68 cm × 30 cm) located in a soundproof room. The behavior was monitored for 10 min using a video tracking system for automation of the behavioral experiments (Multi Conditioning System, TSE Systems, Germany).

We recorded the locomotor activity, expressed as the total distance traveled, the time spent in the center of the arena, and the number of rearings. The ‘center’ was defined as the central part of the arena occupying half of the total surface of the arena. The greater proportion of time spent in the peripheral parts of the arena compared to the center was regarded as a marker of anxiety.

2.8. Data analysis

The following kinetic parameters of serotonin uptake were used to characterize SERT activity: (1) apparent Michaelis constant (Km), characterizing affinity of serotonin transporter (calculated as extracellular serotonin concentration when the velocity of its transport equals to 50% of Vmax); (2) maximal velocity of serotonin transport (Vmax), and
(3) \( \frac{V_{\text{max}}}{K_{\text{m}}} \) ratio representing limiting permeability at low extracellular concentrations of serotonin (apparent serotonin uptake efficiency). SigmaPlot software with Enzyme Kinetics Module (version 10.0, Systat Software Inc., Richmond, CA, USA) was used to calculate the parameters \( V_{\text{max}} \) and \( K_{\text{m}} \).

The anisotropy \( (r) \) was calculated from the fluorescence intensity when both polarizers were vertical \( (k_{\text{v}}) \) and when the excitation was vertical and the emission horizontal \( (k_{\text{h}}) \). Correction for dark values (slits closed) and differential transmission and detection at the two polarizer positions was included:

\[
r = \frac{(k_{\text{v}} - G \cdot k_{\text{h}})}{(k_{\text{v}} + 2 \cdot G \cdot k_{\text{h}})}
\]

where the \( G \) factor was measured using horizontally polarized excitation:

\[
G = \frac{k_{\text{v}}}{k_{\text{h}}}
\]

Data are expressed as the arithmetic means. Standard deviation (SD) was calculated to characterize group variability. Hypothesis testing was performed using Mann–Whitney \( U \) test and by Wilcoxon paired difference test.

**Cholesterol level** was analyzed by two-tailed \( t \)-test comparing simvastatin and control group.

Behavioral analysis: incidence (rearings, number of entrances, percentage of time and distance) was analyzed using the Chi-square test; numerical values were analyzed by an unpaired \( t \)-test.

### 3. Results

**3.1. Membrane microviscosity**

The membrane microviscosity \( (r) \) of erythrocyte ghosts was characterized by the fluorescence anisotropy of membrane fluorescent probes DPH and TMA-DPH (Figs. 2 and 3). Changes in fluorescence anisotropy during treatment with simvastatin were evaluated by the Wilcoxon paired test, i.e. values were compared before treatment (day 1, baseline) with those at day 14 or day 28 of the treatment with simvastatin. Compared to the baseline measurement, the fluorescence anisotropy of both DPH and TMA-DPH was significantly \( (p < 0.05) \) decreased after simvastatin application after 2 as well as 4 weeks (Fig. 2). In the controls, the decrease of fluorescence anisotropy was not significant at day 14 or day 28 compared to day 1.

The ratio of fluorescence anisotropy in the erythrocyte ghosts of rats treated with simvastatin to fluorescence anisotropy of controls was analyzed to eliminate changes that are not related to the effect of simvastatin. A significant decrease in this ratio against the control \( (100\%) \) was found for DPH at days 14 and 28, and for TMA-DPH at day 14 (Fig. 3). A significant increase in the ratio was observed for TMA-DPH at day 28 compared to day 14.

**3.2. Kinetics of platelet serotonin uptake**

The kinetics of platelet serotonin uptake was described by \( V_{\text{max}}, K_{\text{m}} \), and the ratio \( V_{\text{max}}/K_{\text{m}} \) (Fig. 4). Changes in 5-HT uptake during treatment were evaluated by the Wilcoxon paired test, i.e. the values before treatment (baseline) were compared with those at day 14 or day 28 of the treatment with simvastatin. Compared to the baseline measurement (day 1), \( V_{\text{max}} \) was significantly decreased after 4 weeks of application \( (p = 0.043) \). No significant difference in the kinetics of platelet serotonin uptake was found between the control group and the simvastatin-treated group either at day 14 or day 28 of the treatment.

**3.3. Cholesterol levels**

The analysis of cholesterol levels in the brain revealed significant differences between the simvastatin group and the control group in all of the tested brain regions: the amygdala (two-tailed unpaired \( t \)-test; \( t = 12.005; p < 0.001 \)), hippocampus (\( t = 7.957; p < 0.001 \)), and prefrontal cortex (\( t = 6.388; p \text{ value} < 0.0001 \) (Fig. 5).

**3.4. Behavioral analysis**

Analysis of the rats’ behavior in EPM (Fig. 6) showed a significantly longer time spent in the open arms by the simvastatin group than by the control group (Chi-square test, \( p < 0.05 \)). However, we found no differences in ‘anxiety’, expressed as a ratio of the number of entrances to the open arms compared to the entrances to the closed arms. In contrast, we observed a significantly higher number of entrances to the closed arms and also significantly higher ‘impulsivity’, expressed as a sum of the entrances to both the closed and opened arms in the simvastatin group (Chi-square test, \( p < 0.001 \)). One observation data set was
when uptake parameters of rats treated with simvastatin for 2 or 4 weeks were excluded from future analysis [49].

controls (structures in the simvastatin-treated group (Fig. 4.)). Cholesterol was significantly decreased in all structures in the simvastatin-treated group (p < 0.0001) (n = 6) in comparison with controls (n = 6).

4. Discussion

Despite emerging evidence of the role of statins in the central nervous system, there is relatively limited knowledge of the neurobiological mechanisms associated with the behavioral effects of statins. Our results confirmed the cholesterol-lowering effect of simvastatin in the brain and further supported the hypothesis that lowering of plasma cholesterol after simvastatin treatment affects membrane fluidity and the transmembrane transport of serotonin in the brain. Moreover, behavioral analysis showed subtle changes e.g. longer time spent in the open arms and higher number of entrances to the closed arms in the elevated plus maze for the simvastatin group than the control group. The longer time spent in the open arms is traditionally interpreted as an anxiolytic effect but with respect to the results of some of the clinical studies in humans [2–5] we also consider impulsivity as an alternative explanation.

Behavioral changes induced by long-term statin treatment in the animal models were demonstrated in a number of previous studies, both in rats and mice (see below). However, the interpretations of such changes vary across the studies. Frequently, the behavioral changes in the animal model are seen as a result of the anxiolytic or antidepressant effect of statins in various behavioral tests. Citraro et al. [50] demonstrated the effects of long-term treatment with several statins (simvastatin, atorvastatin and pravastatin) on anxiety and depressive-like behavior in WAG/Rij rats (genetic animal model of epileptogenesis and mild depression comorbidity). All three statins reduced the immobility time in forced swimming test (FST) at 6 months of age; however, this effect was not maintained at 10 months of age (5 months after suspension). Similarly, all statins increased both the number of entries and the time spent in the center of the OF test in rats after 1 month of suspension but not after 5 months of suspension. Santos et al. [51] demonstrated both an antidepressant effect in FST and an anxiolytic effect in EPM and OF after co-application of simvastatin (1 or 10 mg/kg/day) and fluoxetine. Wang and al. [30] found both an anxiolytic effect and hyperlocomotion in EPM and OF. Schilling et al. [52] demonstrated behavioral changes of basic exploration in the open field test and moreover cognitive alterations in the Barnes maze and affected startle response. Lin et al. [37] reported that simvastatin (5 or 10 mg/kg/day) can reverse depressive-like states developed in rats exposed to chronic mild stress for 4 weeks. An antidepressant-like effect of simvastatin treatment was demonstrated in a forced swimming test and sucrose preference test; however anxiety-like behavior in OF and EPM was not influenced. In contrast, Klici et al. [53] showed a dose independent effect of statin treatment, whereby doses of 10 and 30 mg/kg, but not 50 mg/kg increased time spent in the open arms of EPM and also reduced the immobility period in a forced swimming test. The dose or period of a treatment can often explain the ambiguity of interpretations in the previously mentioned studies. Therefore, the behavioral effect of simvastatin treatment can also depend on various parameters and different methodologies. In addition, the discrepancies in the literature can be possibly related to different levels of stress occurring during various animal tests of anxiety and depression. Moreover, there can be variances between studies of sensitivity to simvastatin treatment in intact animals and experimental animals exposed to stress during the experiments.

Interestingly, a potential anxiolytic and anti-depressant effect of the statin treatment in the animal model is in contradiction with the findings from some human studies, which report an association between lower cholesterol levels and greater prevalence of depressive symptoms (for a review see [54]). Such ambiguity means it is necessary to search for the precise motivation and mechanisms underlying the behavioral changes observed both in animal and human studies.

In our study, we determined significant differences in behavior between the simvastatin and control groups in the elevated plus maze but not in the open field test. In the elevated plus maze, we observed that the simvastatin group animals spent not only a significantly longer period of time in the open arms but there was also a higher number of entrances to closed arms and a significantly higher overall number of entrances to both arms. This behavior can be viewed as an anxiolytic effect of simvastatin; however, we may ask whether another motivation underlies such behavior. This behavior is not in contradiction with our assumption that simvastatin may lead to impulsive behavior (loss of behavioral inhibition). The elevated plus maze is based on two conflicting motivations, a rodent’s drive to explore a novel environment and its aversion to open spaces. Therefore, the effect of the simvastatin treatment can also be explained in terms of increased risk-taking behavior. Effects on the animal’s behavior require additional testing to better explain the observed behavioral alterations in the experimental tasks. To distinguish precisely the particular mechanisms
underlying behavior, we will carry out more detailed behavioral tests in the following study particularly concerning impulsive behavior using operant tests already used in our laboratory [55–58] and applied for time-interval tasks.

The behavioral changes that we observed in our study correspond with the lowered level of brain cholesterol in the simvastatin group. Lower cholesterol content was demonstrated in all of the tested brain regions i.e. the hippocampus, striatum and frontal cortex. Also, a link

Fig. 6. Analysis of rats' behavior in the elevated plus maze (means ± SEM). We analyzed (1) the percentage of time spent in open arms, closed arms, center of the maze; (2) the number of entrances into the open and closed arms; (3) “anxiety” (expressed as a ratio of the number of entrances to the open arms compared to the entrances to closed arms) and (4) “impulsivity”, expressed as a sum of the entrances both to the closed and opened arms. In the simvastatin-treated group we observed a significantly longer time spent in the open arms, a significantly higher number of entrances to the closed arms and also significantly higher “impulsivity”. White bars — control group (n = 5), one animal was excluded as an outlier (based on CI analysis and Grubb’s test; p < 0.05). Black bars — simvastatin-treated group (n = 6). Asterisk/significant difference (*p < 0.05; ***p < 0.001).
between impairment of serotonin neurotransmission, microviscosity of plasmatic membranes and their cholesterol content is in accordance with our previous research [3,8,13].

Our in vivo experiments show that both SERT functioning in platelets and microviscosity of erythrocyte membranes are influenced by simvastatin. Because SERT activity is regulated by cholesterol [31] and membrane microviscosity strongly depends on the cholesterol content in the membrane [59], one explanation of our findings could be that a simvastatin-induced decrease of plasma cholesterol [13] can be responsible for these effects. An important mechanism for the modulation of SERT activity is the density of the transporter molecules at the cell membrane and their affinity to 5-HT. Kinetic parameters of serotonin uptake, maximum velocity (Vmax) and apparent Michaelis constant (Km) are used to characterize SERT activity. Previous findings indicated that SERT is regulated both by membrane cholesterol [31] and by several regulatory proteins [32].

Membrane microviscosity, expressed by fluorescent anisotropy, demonstrates a significant difference between the control group and the simvastatin-treated group both at day 14 and day 28 of the treatment. Both the core of the bilayer in which DPH was embedded and the surface in which the trimethyl-ammonium derivative of DPH (TMA-DPH) was embedded suggested a lower microviscosity (greater fluidity) in the simvastatin-treated rats than in the rats before treatment. We suppose that this effect is caused by a decrease in membrane cholesterol.

The maximum velocity of serotonin transport was decreased after application of simvastatin compared to the baseline measurement. However, SERT activity quantified by the Vmax/Km ratio was not significantly changed, indicating the existence of a compensatory mechanism related to the SERT activity. The different effects of the 2-week and 4-week treatments with simvastatin on both membrane microviscosity and serotonin uptake support the existence of adaptive changes in the serotonergic system. The effect of the 2-week application of simvastatin on serotonin uptake in rat platelets was found to be similar to the effect of depletion of membrane cholesterol in human embryonic kidney cells. It resulted in a decrease in SERT activity both due to a loss of affinity of substrate and ligand binding and a concomitant reduction of the maximum transport rate [31]. Moreover, it is of interest that the mild decrease in SERT activity (increase in Km and decrease in Vmax) after a short-term application of simvastatin is smaller but similar to the effects of serotonin reuptake inhibitors used as antidepressants [60]. However, it is difficult to interpret the physiological role of the observed simvastatin-induced changes in the serotonergic system because stress-induced changes cannot be excluded in our study.

Nevertheless, our data suggest the significant effect of simvastatin treatment on serotonergic neurotransmission. Consistent with this, both simvastin and lovastatin potentiated the anxiolytic-like and antidepressant-like effects of fluoxetine, a selective serotonin reuptake inhibitor, in the EPM [51] and forced swimming test [38]. Interestingly, Kilic et al. [53] reported that simvastatin also potentiated the antidepressant effect of sertraline, also a selective serotonin reuptake inhibitor, but did not change the effect of amitriptyline, a tricyclic antidepressant. The results suggest that simvastatin can influence behavior in an analogical way relating to SSRI drugs.

5. Conclusion

We demonstrated that long-term simvastatin application results in complex physiological and behavioral alterations. Such changes are difficult to interpret and it is necessary to carry out additional detailed studies to interconnect cellular, molecular and behavioral aspect levels.

In conclusion, our results support the hypothesis that lowering of plasma cholesterol with simvastatin affects membrane fluidity and different cellular processes regulated by cholesterol, such as the transmembrane transport of serotonin. It remains to be determined precisely whether these effects of simvastatin treatment are related to its desired or adverse effects.

Author contribution

All of the authors contributed extensively to the project presented in this paper and discussed the results and implications. All of the authors made comments on the manuscript at all stages. TN, KV, JV and ZF made substantial contributions to the conception of the paper and are responsible for formulation of the hypothesis, PK developed chemical analytical tools and analyzed the cholesterol content, ZF, NS and JH analyzed the serotonergic functions, and AS critically reviewed and edited the manuscript.

The behavioral experiments were performed entirely at the Institute of Physiology, CAS, National Institute of Mental Health and Charles University covered theoretical works and writing the manuscript. The analyses were performed at Charles University and the Department of Organic Technology, Institute of Chemical Technology.

Disclosure statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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