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The impact of capsaicinoids on APP processing in Alzheimer's disease in SH-SY5Y cells

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The vanilloid capsaicin is a widely consumed spice, known for its burning and “hot” sensation through activation of TRPV1 ion-channels, but also known to decrease oxidative stress, inflammation and influence tau-pathology. Beside these positive effects, little is known about its effects on amyloid-precursor-protein (APP) processing leading to amyloid- β (A β), the major component of senile plaques. Treatment of neuroblastoma cells with capsaicinoids (24 hours, 10 μ M) resulted in enhanced A β -production and reduced A β -degradation, leading to increased A β -levels. In detailed analysis of the amyloidogenic-pathway, both BACE1 gene-expression as well as protein-levels were found to be elevated, leading to increased β -secretase-activity. Additionally, γ -secretase gene-expression as well as activity was enhanced, accompanied by a shift of presenilin from non-raft to raft membrane-domains where amyloidogenic processing takes place. Furthermore, impaired A β -degradation in presence of capsaicinoids is dependent on the insulin-degrading-enzyme, one of the major A β -degrading-enzymes. Regarding A β -homeostasis, no differences were found between the major capsaicinoids, capsaicin and dihydrocapsaicin, and a mixture of naturally derived capsaicinoids; effects on Ca²⁺-homeostasis were ruled out. Our results show that in respect to Alzheimer's disease, besides the known positive effects of capsaicinoids, pro-amyloidogenic properties also exist, enhancing A β -levels, likely restricting the potential use of capsaicinoids as therapeutic substances in Alzheimer's disease.

Alzheimer's disease (AD) is the most common neurodegenerative disorder, characterized by degeneration of neurons in multiple brain regions, resulting in progressive memory loss and cognitive failure. Extracellular senile plaques, consisting of aggregated amyloid- β (A β) peptides, and intracellular neurofibrillary tangles, composed of abnormally hyperphosphorylated microtubule-associated tau-proteins, are the main histopathological hallmarks of AD^{1,2}. A β peptides are generated by sequential proteolytic processing of the amyloid precursor protein (APP), a large type-I transmembrane protein³. A β generation involves shedding of the extracellular/luminal APP domain by β -secretase BACE1^{4–6}, generating β -secreted APP (sAPP β) and a membrane-tethered C-terminal fragment, called β -CTF or C99. β -CTF is further cleaved by γ -secretase within its transmembrane domain, releasing A β peptides with variable C-termini, mainly A β 38, A β 39, A β 40 and A β 42 peptides^{7,8}, and the APP intracellular domain (AICD) (Fig. 1a). γ -secretase is a heterotetrameric protein complex consisting of anterior-pharynx-defective 1a or b (APH1a, APH1b), presenilin 1 or 2 (PS1, PS2), nicastrin and presenilin-enhancer 2 (PSEN2)^{9,10}. The aspartyl-proteases PS1 and/or PS2 are the catalytically active components of the γ -secretase complex^{11–13}. Besides the A β producing amyloidogenic pathway, APP can be processed in a non-amyloidogenic pathway by α -secretases, cleaving APP within the A β domain and thus preventing the release of neurotoxic A β peptides^{14–17}. Similar to β -secretase cleavage of APP, α -secretase shedding also results in a secreted fragment, namely sAPP α . Total A β levels are not only dependent on the A β anabolism by the APP processing machinery, but also on A β catabolism via different mechanisms including proteolytic elimination by the main A β -degrading enzymes neprilysin

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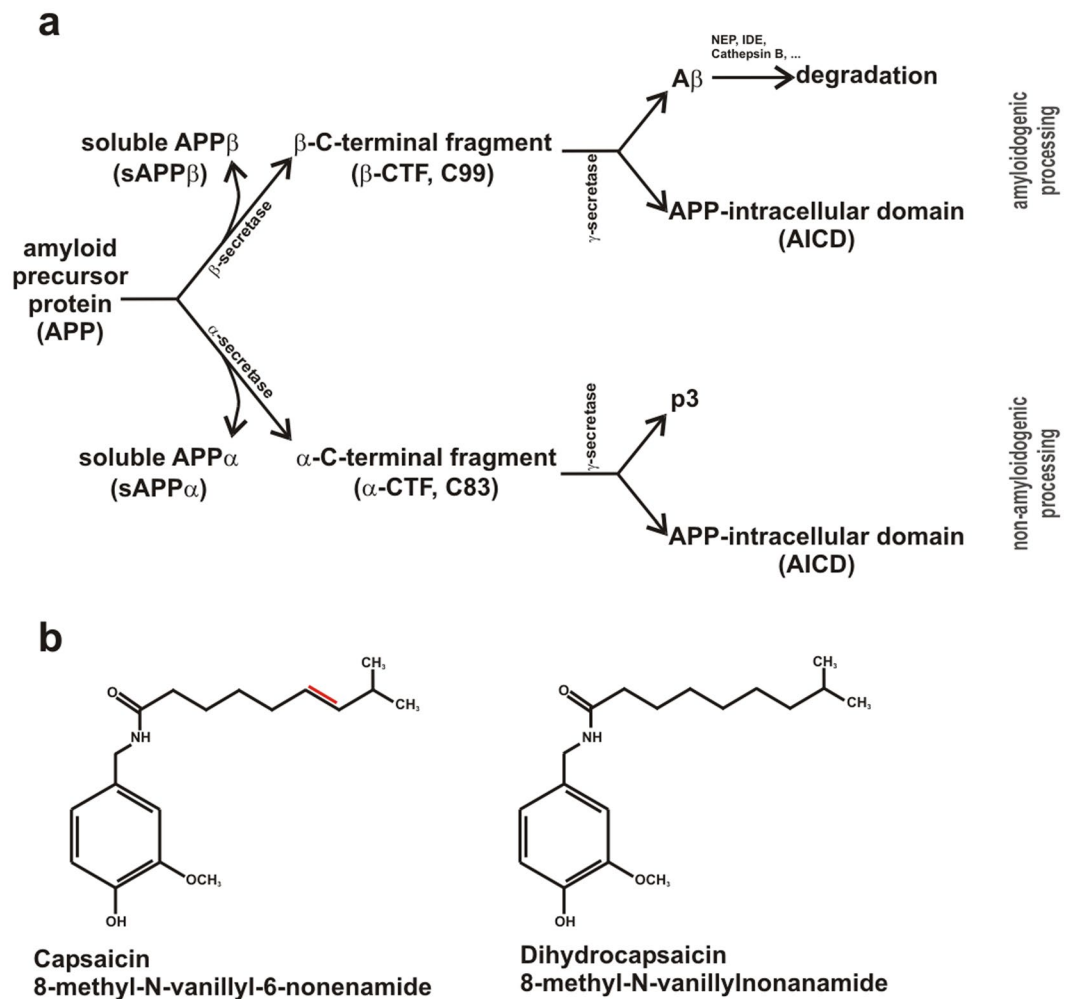


Figure 1. (a) Schematic illustration of amyloidogenic and non-amyloidogenic processing of the amyloid precursor protein (APP). (b) Chemical structures of capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonanamide) with structural differences highlighted in red.

(NEP) and insulin-degrading enzyme (IDE)^{18,19}. APP and the APP-cleaving secretases are ubiquitously expressed in the human body and several physiological functions of the APP cleaving products have been described^{20–26}. Furthermore, the γ -secretase complex cleaves several other substrates beside APP^{27–29}. Current research focuses on modulating instead of completely inhibiting the pathways involved in A β generation to prevent severe side effects due to the physiological function of other secretase-mediated pathways. Several A β -lowering dietary components, bioactive compounds or supplements have been described, e.g. DHA, vitamin D and vitamin D analogues, polyphenols, caffeine and some phytosterols^{30–36}. Recently, capsaicin, found in *Capsicum* species and spices consumed worldwide, has been proposed to be potentially beneficial in respect to tau alterations of AD in patients suffering from type 2 diabetes, known as risk factor for the development of AD^{37,38}. Furthermore, it has been described that capsaicin improves cold water stress-induced AD-like pathological and cognitive impairments in rats³⁹. In respect to the amyloidogenic processing and A β homeostasis the current literature is controversial. It has been reported that capsaicin consumption reduces blood A β levels in humans⁴⁰. In contrast to the potential beneficial effects in respect to AD, a previous study described that capsaicin might promote the amyloidogenic pathway of APP processing⁴¹.

Capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide) is the primary and naturally occurring ingredient found in *Capsicum* species, responsible for the piquancy of chili peppers. Structurally, capsaicin belongs to a group of chemicals known as vanilloids with three important regions, an aromatic head, an amide linkage and a hydrophobic tail (Fig. 1b). Beside capsaicin, more than twenty capsaicinoids were identified in *Capsicum* species⁴², varying in the length and degree of saturation in the aliphatic side chain. Capsaicin and dihydrocapsaicin (DHC) are the most prominent capsaicinoids in *Capsicum* species, accounting for 80% to 90% of the capsaicinoids⁴². Capsaicinoids possess pleiotropic pharmacological and physiological activities, including anti-inflammatory, anti-apoptotic, anti-oxidant, anti-cancer, anti-obesity, neuro-protective and analgesic functions^{43–46}. Furthermore, capsaicin has been shown to have beneficial effects on cardiovascular, gastrointestinal and dermatological diseases^{47–49}. Capsaicin is a highly selective agonist of the transient receptor potential channel of the vanilloid receptor family, subtype 1 (TRPV1), a Ca²⁺ permeable nonselective cation ligand-gated channel

highly expressed in unmyelinated C-type sensory nerve fibers, in small-diameter sensory neurons of the dorsal root, trigeminal and vagal ganglion^{50–52} as well as in the hippocampus, striatum, hypothalamus and cerebellum of human and rat brain⁵³. TRPV1 activation causes influx of extracellular calcium, resulting in elevated intracellular calcium, which is involved in a variety of physiological processes⁵⁴. TRPV1 activation in the central nervous system contributes to basic neuronal functions including resting membrane potential, neurotransmitter release, synaptic plasticity and hippocampal memory function⁵⁵. Also, several TRPV1-independent effects of capsaicin have been described in the past^{56–59}.

As capsaicin is widely consumed as a nutritional compound or spice and the effects in respect to AD are either controversial or not investigated, we systematically examined the effects of the two major capsaicinoids, capsaicin and DHC, as well as a naturally occurring mixture on APP processing and A β homeostasis.

Materials and Methods

Chemicals and reagents. Capsaicin, dihydrocapsaicin (DHC) (analytical standards), natural occurring capsaicin and, if not stated otherwise, all other chemicals used in this study were acquired from Merck former Sigma-Aldrich (Darmstadt, Germany).

Cell culture. Cells of the human neuroblastoma SH-SY5Y cell line were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum (FCS, GE Healthcare Life Sciences, Chalfont St. Giles, United Kingdom) and 0.1% non-essential amino acid solution (MEM). For the African green monkey fibroblast-like cell line COS7, culture medium contained DMEM and 10% FCS. For the stably transfected cell lines SH-SY5Y/COS7 APP⁶⁹⁵, which overexpress the major neuronal APP isoform in humans, APP⁶⁹⁵, and for stably transfected SH-SY5Y C99 cell line⁶⁰, which overexpress the β -secretase cleavage product, β -CTF, 400 μ g/mL hygromycin B (PAN-Biotech, Aidenbach, Germany) were added to the media. For the murine neuroblastoma cell line (N2a), culture medium consisted of DMEM, 10% FCS, 0.1 mM MEM, 0.1 mg/mL Streptomycin, 100 U/ml Penicillin, 2 mM L-Glutamine and 1 mM sodium pyruvate. For N2a cells with IDE knock-down, promoted by SureSilencing shRNA plasmids (Qiagen, Hilden, Germany) regarding to manufacturer's protocol and⁶¹, 400 μ g/mL Hygromycin B were added to the media. Knock-down efficiency is shown in Supplemental Fig. S4.

Incubations. *Cell culture.* Dependent on the following experiments, FCS in DMEM was reduced to 1%, 16 h prior incubation. FCS was reduced to elucidate the potential effect of capsaicinoids on cholesterol or other lipids being also present in FCS. Capsaicin, DHC, and natural compound were incubated in a concentration of 10 μ M, Ruthenium Red was incubated at 2 μ M with or without 10 μ M capsaicin for 24 h (8 + 16h), whereas controls were treated with HPLC-grade ethanol (Fisher Scientific, Schwerte, Germany) as a solvent control for the capsaicinoids and HPLC-grade water as solvent control for Ruthenium Red.

Cell lysates. Cells were lysed using 150 mM NaCl (VWR Chemicals, Radnor, PA, USA), 50 mM Tris/HCl pH 7.4, 2 mM EDTA (Carl Roth, Karlsruhe, Germany), 1% NP-40, 1% Triton X-100 lysis buffer supplemented with protease inhibitor (Roche Diagnostics, Basel, Switzerland). Afterwards, the protein concentrations were determined by bicinchoninic acid assay (BCA) as described earlier⁶² and adjusted to equal protein concentrations for following experiments.

Cell homogenates for isolated membranes and raft fractions. Cultivated SH-SY5Y cells were washed two times with ice-cold PBS for membrane isolation, and MBS (25 mM MES, 150 mM NaCl, 0.1% Triton X-100, pH 6.5) for raft isolation. Homogenates for membrane isolation were collected in 150 μ L sucrose buffer (10 mM Tris/HCl, 200 mM sucrose, 1 mM EDTA pH 7.5). Raft-homogenates were solubilized in 500 μ L MBS buffer. The solutions were then homogenized via Minilys (Peqlab, Erlangen, Germany) for 20 seconds on maximum intensity. Afterwards raft-homogenates were further homogenized via a 24 G cannula. Protein amount was adjusted to 5 mg/mL for rafts and 2 mg/mL for membrane isolation. The latter were then centrifuged at 900 rcf for 10 min at 4 $^{\circ}$ C and supernatants containing postnuclear fractions (PNFs) were collected³⁴. Homogenates were stored at -80° C.

Preparation of isolated membranes. PNFs were incubated with 10 μ M capsaicin, natural compound or solvent control at 37 $^{\circ}$ C for 20 min while shaking. Further preparation was performed as described in⁶³. For a detailed description see also supplemental information. Before measuring secretase activity, incubation was performed again as described above.

Lipid raft preparation. Lipid raft preparation was performed as described in³². For a detailed description see also supplemental information.

Western blot (WB) experiments. BACE1 and PS1 protein levels were analyzed in cell lysates, whereas for the determination of the total protein levels of the secreted proteins A β , sAPP α , and sAPP β growth media were used. All samples were adjusted to the same protein amount via BCA. Antibodies and dilutions used in this study are: W02 antibody for the detection of β -CTF and total A β (5 μ g/mL; Millipore, Billerica, MA, USA), anti-human sAPP β (1:50; IBL America, Minneapolis, MN, USA), anti-human BACE1 B0806 (1:1000; Merck, former Sigma-Aldrich, Darmstadt, Germany), anti-human PS1 sc-7860 (Santa Cruz, Dallas, Texas, US), anti-rabbit IgG HRP Conjugate W4011 (1:5000; Promega, Mannheim, Germany) and anti-mouse P0260 (Dako, Hamburg, Germany). For detection of proteins, the enhanced chemiluminescence (ECL)-method (Perkin Elmer, Rodgau-Jügesheim, Germany) was used. The quantification was performed densitometrically with Image Gauge V3.45 software (Fujifilm, Düsseldorf, Germany).

Immunoprecipitation. For detection of A β and β -CTF, immunoprecipitation of equal volumes of conditioned media or cell lysates, adjusted to the same protein amount, was performed as described in⁶⁴. Precipitates were further used for WB experiment. For a detailed description see also supplemental information.

Determination of total A β -degradation in N2a wt and N2a IDE knock down cells. N2a wt/IDE knock-down cells were reduced in FCS to 1%/DMEM 16 h before incubation. To determine the total degradation of A β , cells were incubated with 10 μ M capsaicin or HPLC-grade ethanol as control for 18 h. Then, incubation medium was replaced now containing not only capsaicin or ethanol, but also human synthetic A β 40 peptide (Bachem, Bubendorf, Switzerland) (0.5 μ g/mL). After 6 h, non-degraded human A β was detected by WB analysis using W02 antibody.

Secretase activity assays. β - and γ -secretase activities in living SH-SY5Y wild type cells were analyzed as described in⁶⁵. Activities of both secretases in isolated membranes and raft fractions were examined as described in⁶⁶. For detailed descriptions of these methods see also supplemental information.

Quantitative real-time polymerase chain reaction (RT-PCR) experiments. Gene expression analysis was performed as described in⁶⁷. In this study the following primers were used: actin (*ACTB*): forward 5'-CTT CCT GGG CAT GGA GTC-3', reverse 5'-AGC ACT GTG TTG GCG TAC AG-3'; anterior-pharynx-defective 1 homolog A (*APH1A*): forward 5'-GCC TCT GTG GTC TGG TTC AT-3', reverse 5'-TCT GCC TTC TTA AGC AGC TTG T-3'; β -site APP-cleaving enzyme 1 (*BACE1*): forward 5'-GCA GGG CTA CTA CGT GGA GA-3', reverse 5'-TAG TAG CGA TGC AGG AAG GG-3'; nicastrin (*NCSTN*): forward 5'-CTG TAC GGA ACC AGG TGG AG-3', reverse 5'-GAG AGG CTG GGA CTG ATT TG-3'; presenilin1 (*PSEN1*): forward 5'-CTC AAT TCT GAA TGC TGC CA-3', reverse 5'-GGC ATG GAT GAC CTT ATA GCA-3'; presenilin2 (*PSEN2*): forward 5'-GAT CAG CGT CAT CGT GGT TA-3', reverse 5'-GGA ACA GCA GCA TCA GTG AA-3'; presenilin enhancer (*PSENEN*): forward 5'-CAT CTT CTG GTT CTT CCG AGA G-3', reverse 5'-AGA AGA GGA AGC CCA CAG C-3' (Eurofins MWG Operon, Eberberg, Germany). Results were normalized to actin and the $2^{-(\Delta\Delta C_t)}$ method was used to calculate expression changes.

Cholesterol concentration. Cholesterol concentration was measured by using the Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Lactate dehydrogenase (LDH) activity assay. To measure the cytotoxicity of the capsaicin incubation, Cytotoxicity Detection Kit (LDH) from Roche (Basel, Switzerland) was used according to the manufacturer's instructions.

Fluorescence-based Ca²⁺ imaging. To monitor intracellular calcium levels after capsaicin application, a calcium imaging setup was used as described in⁶⁸.

SH-SY5Y APP⁶⁹⁵ cells were incubated on glass coverslips with 1 μ M Fura 2-AM at room temperature for 25 min. A self-built perfusion chamber was used with low volume and high solution exchange rate. The external Ca²⁺ Ringer solution contained (in mM): 145 NaCl, 4 KCl, 2 MgCl₂, 10 glucose, 10 Hepes and 1.2 Ca²⁺ Ringer (pH 7.4 with NaOH). After washing the cells twice, 10 μ M capsaicin was added. Ca²⁺ images were recorded before, during, and after application of capsaicin. For analysis TILLVision followed by Igor software was used.

As a proof of principal, SH-SY5Y APP⁶⁹⁵ cells were transiently transfected with human *TRPV1* and *GFP* as negative control according to manufacturer's guidelines 24 h before imaging.

Thioflavin T based A β aggregation assay. To measure aggregation of A β , we modified the thioflavin T based assay described in⁶⁹. 500 μ g A β 42 peptide (generous gift of B. Penke, Szeged, Ungary) was dissolved and incubated for 24 h in 1 mL hexafluoro-2-propanol (HFIP) at 4 °C. Monomerized peptides were then dried via SpeedVac (Savant DNA110, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. Afterwards dried peptides were resolved in 100 μ l ammonium hydroxide, sonicated for 30 min on ice, and filtered via centrifugation for 90 min at 13,900 rpm and 8 °C in Amicon Ultra-0.5 mL Filters. Final A β concentrations were detected via UV-Vis-spectrophotometer (NanoDropTM 8000, Thermo Fischer Scientific, Waltham, MA, USA) absorbance measurement at 280 nm. 10 μ M of freshly monomerized A β 42 was incubated with 15 μ M thioflavin T on a black 96 well quartz microplate (HellmaTM, Müllheim, Germany) in 100 μ l total volume per well and in the presence of 10 μ M natural compound capsaicin or ethanol as solvent control, both conditions were performed in presence of 10 μ M phosphatidylcholine 16:0. Measurement was performed with an infinite M1000 Pro (Tecan, Crailsheim, Germany) at 37 °C for at least 3 days with the following wavelengths: excitation at 450 \pm 5 nm and fluorescence detection at 482 \pm 5 nm.

IDE activity assay. IDE enzyme activity was analyzed as described in⁷⁰ with minor modifications. Anti-IDE antibody ST1120 (Merck) was diluted in 1xPBS (5 μ g/ml) and coated on a Nunc MaxiSorp 96-well plate for 24 hours at room temperature. After washing the plate five times with 1x PBS containing 0.5% Tween-20, blocking was performed by incubating 10% fatty acid free bovine serum albumin in 1x PBS for two hours at room temperature. Afterwards, plate was washed three times and lysates were incubated for one hour at 20 °C and 150 rpm, followed by five wash steps. After a pre-incubation with assay buffer (50 mM Tris-HCl pH 7.4, 1 M NaCl, 10 μ M MgCl₂, β -secretase inhibitor II (565749, Merck), γ -secretase inhibitor IV (565761, Merck), complete protease-inhibitor without EDTA) for 15 minutes at room temperature, the fluorogenic peptide substrate Mca-RPPGFSAFK(Dnp)-OH (10 μ M) was added and fluorescent was measured using a Safire² Fluorometer

(Tecan, Crailsheim, Germany) at an excitation wavelength of 320 ± 10 nm, and an emission wavelength of 405 ± 10 nm.

Statistical analysis. All quantified data represent an average of at least three independent experiments. Error bars represent standard deviation of the mean. Normal distribution was tested by Kolmogorov-Smirnow and Shapiro-Wilk tests. Statistical significance was determined by two-tailed Student's *t* test for comparing two parameters or ANOVA (Games-Howell and Bonferroni *post hoc*) tests in case of multiple parameter comparison. Significance was set at $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Results

Capsaicinoids increase amyloidogenic APP processing. To examine the effect of capsaicinoids on APP processing, we analyzed the most predominant forms present in *Capsicum*, capsaicin (trans-8-ethyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (DHC), as well as the natural compound comprising beside capsaicin and DHC several other capsaicinoids⁴². The aliphatic side chains of capsaicin and DHC have an identical length but vary in respect to a double bond which is present in capsaicin but is lacking in DHC (Fig. 1b). As cellular systems we used three different stably transfected cell lines: human neuroblastoma SH-SY5Y cells and COS7 cells stably expressing APP⁶⁹⁵, the most abundant APP isoform found in neurons⁷¹, and SH-SY5Y cells stably expressing the truncated APP construct C99. C99 represents the C-terminal fragment of β -secretase cleaved APP and only requires cleavage by γ -secretase to release A β ⁶⁰. Cells were incubated for 24 hours in presence of $10 \mu\text{M}$ capsaicinoids and A β generation was monitored by a highly sensitive A β -immunoprecipitation and Western blot (WB) analysis⁷². All analyzed substances revealed a very similar increase in the A β levels in APP⁶⁹⁵ transfected SH-SY5Y cells upon capsaicin treatment: In presence of pure capsaicin, A β levels were significantly elevated to 136.1%; in cells incubated with the natural compound A β levels were significantly increased to 138.1% and DHC treated cells showed a significant elevation to 137.1% of secreted A β compared to cells treated with the solvent control ethanol (capsaicin: $136.1\% \pm 6.2\%$, $p \leq 0.001$; natural compound: $138.1\% \pm 7.8\%$, $p = 0.003$; DHC: $137.1\% \pm 10.9\%$, $p = 0.037$) (Fig. 2a). Notably, no cytotoxicity was observed under these experimental conditions (Supplemental Fig. S1). In line with these findings capsaicinoid treated APP⁶⁹⁵ expressing COS7 cells, an immortalized cell line of the African green monkey, also revealed an increase in the A β levels. In these cells, capsaicin treatment led to a significant A β elevation to 124.5% and the natural compound caused a significant increase in the A β levels to 127.2% (capsaicin: $124.5\% \pm 5.6\%$, $p = 0.002$; natural compound: $127.2\% \pm 3.6\%$, $p = 0.001$) (Fig. 2b). The capsaicinoid induced increases in the A β levels were slightly reduced compared to APP⁶⁹⁵ transfected SH-SY5Y cells, which might be explained by the fact that in non-neuronal cells APP is primarily cleaved by α -secretase whereas neuronal cells show an elevation in β -secretase APP cleavage^{73–75}. No significant differences between the single compounds were observed for both APP⁶⁹⁵ expressing SH-SY5Y and COS7 cells, as determined by ANOVA analysis. In order to examine the effect of capsaicin on γ -secretase processing independent of β -secretase cleavage, we analyzed SH-SY5Y cells stably transfected with β -CTF/C99. Capsaicin treated β -CTF/C99 expressing SH-SY5Y cells also showed a significant increase in the A β levels to 126.0% (capsaicin: $126.0\% \pm 9.7\%$, $p = 0.020$) (Fig. 2c), indicating that γ -secretase processing of APP is affected by capsaicinoids. However, as the effect strength for secreted A β levels observed in β -CTF/C99 expressing SH-SY5Y cells was slightly reduced compared to APP⁶⁹⁵ expressing SH-SY5Y cells, one can assume that capsaicin also affects β -secretase processing of APP. Therefore, we analyzed the APP cleavage products generated by β -secretase processing, β -secreted APP (sAPP β) and β -CTF. In presence of capsaicin, β -CTF was increased to 147.2% in cell-lysates of APP⁶⁹⁵ transfected SH-SY5Y cells, though the observed increase did not reach significance (β -CTF: $147.2\% \pm 12.56\%$, $p = 0.057$) (Fig. 2d). However, detection of sAPP β in the conditioned medium of capsaicin treated APP⁶⁹⁵ expressing SH-SY5Y cells showed a significant increase of sAPP β to 154.2% (sAPP β : $154.2\% \pm 2.9\%$, $p \leq 0.001$) (Fig. 2d), indicating elevated β -secretase processing of APP in presence of capsaicin. Besides increasing β -secretase processing of APP, capsaicin also slightly but significantly elevated the generation of sAPP α to 113.7% ($\pm 3.2\%$, $p = 0.017$) (Fig. 2e).

Capsaicinoids affect β -secretase activity by elevated BACE1 gene expression. The molecular mechanism of the observed capsaicin induced elevated β -secretase cleavage of APP was further examined in neuroblastoma cells by analyzing BACE1 protein levels, gene expression and by performing β -secretase assays in living cells and purified membranes. In presence of capsaicin the BACE1 protein levels were significantly increased to 116.5% (capsaicin: $116.5\% \pm 5.9\%$, $p = 0.0382$). Cells incubated with the natural compound or DHC showed a significant increase in BACE1 protein levels to 131.5% and 137.3%, respectively (natural compound: $131.5\% \pm 5.4\%$, $p = 0.021$; DHC: $137.3\% \pm 10.7\%$, $p = 0.006$) (Fig. 3a). To validate these findings, we analyzed BACE1 gene transcription by real-time (RT)-PCR analysis. All tested capsaicinoids caused a significant increase in BACE1 mRNA levels. RT-PCR analysis of capsaicin treated cells showed a significant increase to 117.9% in BACE1 gene transcription (capsaicin: $117.9\% \pm 0.06\%$, $p = 0.005$), which is in line with the observed increase in the BACE1 protein levels to 116.5%. BACE1 mRNA levels of cells incubated with the natural compound revealed a significant increase to 135.5% and DHC elevated BACE1 gene expression to 182.7% (natural compound: $135.5\% \pm 18.2\%$, $p = 0.039$; DHC: $182.7\% \pm 30.8\%$, $p = 0.009$) (Fig. 3b). However, the observed differences between BACE1 expression as well as in protein levels between the single capsaicinoids did not reach significance using ANOVA analysis. To further strengthen the involvement of β -secretase processing in elevating total A β levels in presence of capsaicinoids, we first measured β -secretase activity in living cells. Capsaicin treated SH-SY5Y cells showed a significant increase in β -secretase activity to 127.1% and cells treated with the natural compound elevated β -secretase activity to 155.5% (capsaicin: $127.1\% \pm 9.3\%$, $p = 0.042$; natural compound: $155.5\% \pm 10.4\%$, $p = 0.001$) (Fig. 3c). Again, ANOVA analysis did not reveal a significant difference between capsaicin and the natural compound. In accordance to the observed elevation in β -secretase activity in living SH-SY5Y cells in presence of capsaicinoids, living HEK cells as well as living N2a cells treated with the natural compound also showed

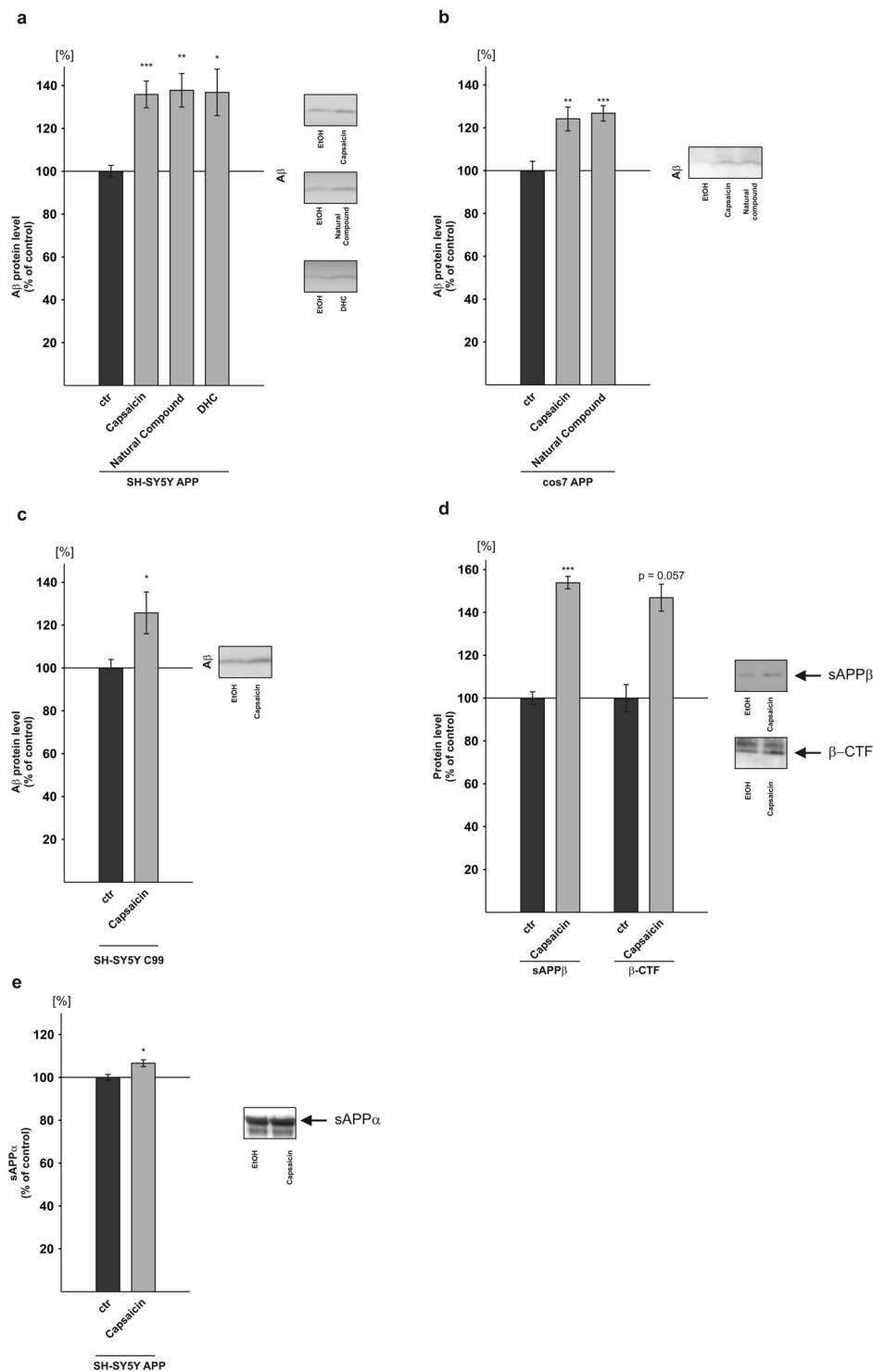


Figure 2. Effect of capsaicinoids on amyloidogenic processing of APP in different cell lines. **(a)** Capsaicin, natural compound and DHC were incubated on SH-SY5Y APP⁶⁹⁵ ($n \geq 9$) cells and A β protein levels were analyzed in comparison to control treated cells by WB. **(b)** COS7 APP⁶⁹⁵ cells were treated with capsaicin and natural compound and levels of A β were determined ($n \geq 4$). **(c)** Protein levels of A β were examined after incubation of capsaicin in SH-SY5Y C99 cells ($n = 14$). **(d)** sAPP β and β -CTF protein levels were analyzed in SH-SY5Y APP⁶⁹⁵ cells after capsaicin treatment ($n = 3$). **(e)** Protein levels of sAPP α were analyzed in SH-SY5Y APP⁶⁹⁵ cells after capsaicin treatment in comparison to solvent control by WB ($n = 5$). Representative signals are illustrated on the right, respectively. Error bars represent the standard error of the mean. Asterisks show the statistical significance calculated by unpaired Student's t test or ANOVA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

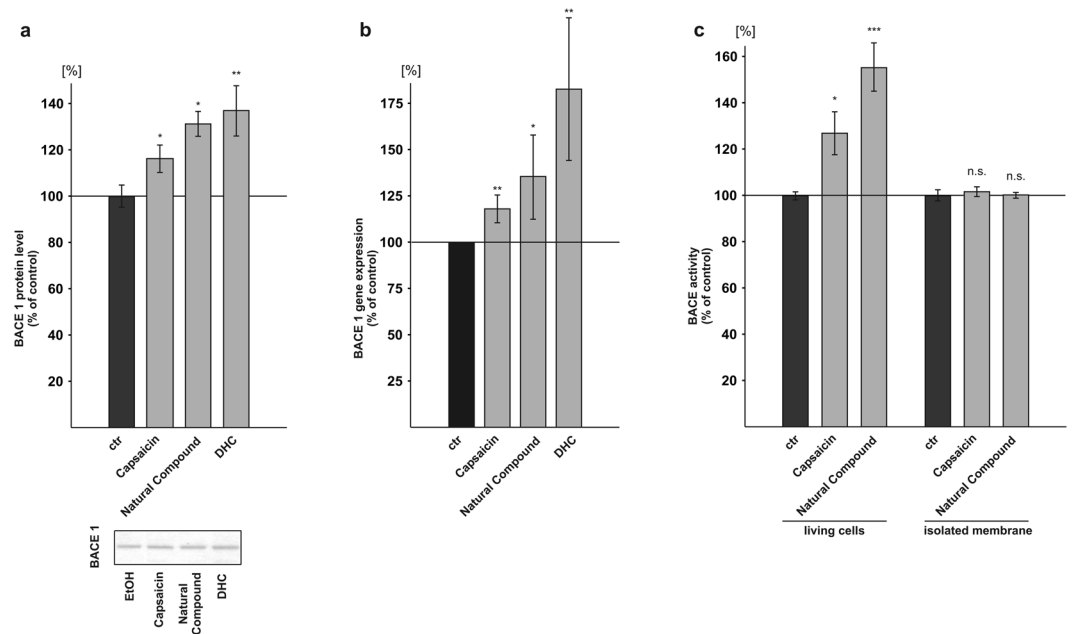


Figure 3. Influence of capsaicinoids on β -secretase. **(a)** Protein levels of BACE1 after incubation of SH-SY5Y APP⁶⁹⁵ cells with capsaicin, natural compound and DHC examined by WB ($n \geq 3$). Representative signals are illustrated on the right. **(b)** BACE1 gene expression analysis by RT-PCR after capsaicinoid treatment in SH-SY5Y cells ($n \geq 4$). **(c)** β -secretase activity in living cells ($n = 10$) and in isolated membranes ($n = 3$) of capsaicin and natural compound incubated SH-SY5Y wt cells. Error bars represent the standard error of the mean. Asterisks show the statistical significance calculated by unpaired Student's t test or ANOVA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

an increase in β -secretase activity to 124.6% and 119.4% respectively, resulting in a mean β -secretase activity enhancement for all analyzed cell lines to 133.2% ($\pm 11.3\%$, $p = 0.042$) (see Supplemental Fig. S5). In contrast to living cells, we observed no effect of capsaicin and the natural compound, when these compounds were incubated on isolated membranes, measuring a potential direct effect on β -secretase activity (Fig. 3c).

Influence of capsaicinoids on γ -secretase activity. As we observed an increase in total A β levels in presence of capsaicinoids by the use of C99 expressing cells, we next analyzed PS1 protein levels. The PS1 protein levels were significantly increased to 144.3% in presence of capsaicin (capsaicin: $144.3\% \pm 11.9\%$, $p = 0.001$). Cells treated with the natural compound or DHC also revealed a significant increase in PS1 protein levels to 132.0% and 152.7%, respectively (natural compound: $132.0\% \pm 7.3\%$, $p = 0.024$; DHC: $152.7\% \pm 7.5\%$, $p \leq 0.001$) (Fig. 4a). In line with elevated PS1 protein levels, capsaicin, the natural compound as well as DHC also significantly increased *PSEN1* mRNA expression as determined by RT-PCR (Table 1). The averaged expression of all γ -secretase components revealed a significant increase to 127.2% for capsaicin, to 142.5% for the natural compound and to 165.7% for DHC (capsaicin: $127.2\% \pm 13.4\%$, $p = 0.050$; natural compound: $142.5\% \pm 7.9\%$, $p \leq 0.001$; DHC: $165.7\% \pm 12.3\%$, $p \leq 0.001$) (Fig. 4b). The RT-PCR results for the single γ -secretase components including *APH1a*, *PSEN1* (PS1), *PSEN2* (PS2), *PSENE1* (presenilin enhancer) and *NCSTN* (nicastrin) are shown in Table 1. Similar to the determination of β -secretase activity, we examined γ -secretase activity in living cells as well as in purified membranes. Capsaicin significantly increased γ -secretase activity in living cells to 128.0% (capsaicin: $128.0\% \pm 7.3\%$, $p = 0.026$). Living cells incubated with the natural compound revealed a significant increase in γ -secretase activity to 135.2% (natural compound: $135.2\% \pm 2.3\%$, $p = 0.007$) (Fig. 4c). The determination of γ -secretase activity in living HEK as well as N2a cells in presence of the natural compound also showed an increase to 117.5% and 142.3% respectively, resulting in a mean γ -secretase activity elevation for all analyzed cell lines to 131.7% ($\pm 7.4\%$, $p = 0.013$) (see Supplemental Fig. S5). Similar to the effect of capsaicinoids on β -secretase activity in isolated membranes, we observed no significant direct effect of capsaicin and the natural compound on γ -secretase enzyme activity (Fig. 4c). Capsaicin and the natural compound only trended to elevate γ -secretase activity directly. As amyloidogenic APP processing has been shown to take place in lipid raft microdomains^{76–78}, we analyzed γ -secretase processing in raft and non-raft microdomains. To determine raft localization of PS1, neuroblastoma cells incubated with capsaicin or solvent control were solubilized in Triton X-100 and separated by buoyant density centrifugation. Raft and non-raft membrane microdomains were identified by immunoblotting with flotillin, a raft marker protein, and cadherin for non-raft fractions (Supplemental Fig. S3). Flotillin-positive fractions were pooled and analyzed for PS1 protein levels. The PS1 protein levels were significantly increased to 137.1% in lipid raft microdomains, whereas the PS1 protein levels were reduced to 85.7% in pooled non-raft cadherin-positive fractions (PS1 protein rafts: $137.1\% \pm 2.9\%$, $p \leq 0.001$; PS1 protein non-rafts: $85.7\% \pm 3.5\%$, $p = 0.044$) (Fig. 4d), indicating a shift of PS1 from non-raft to raft membrane microdomains in

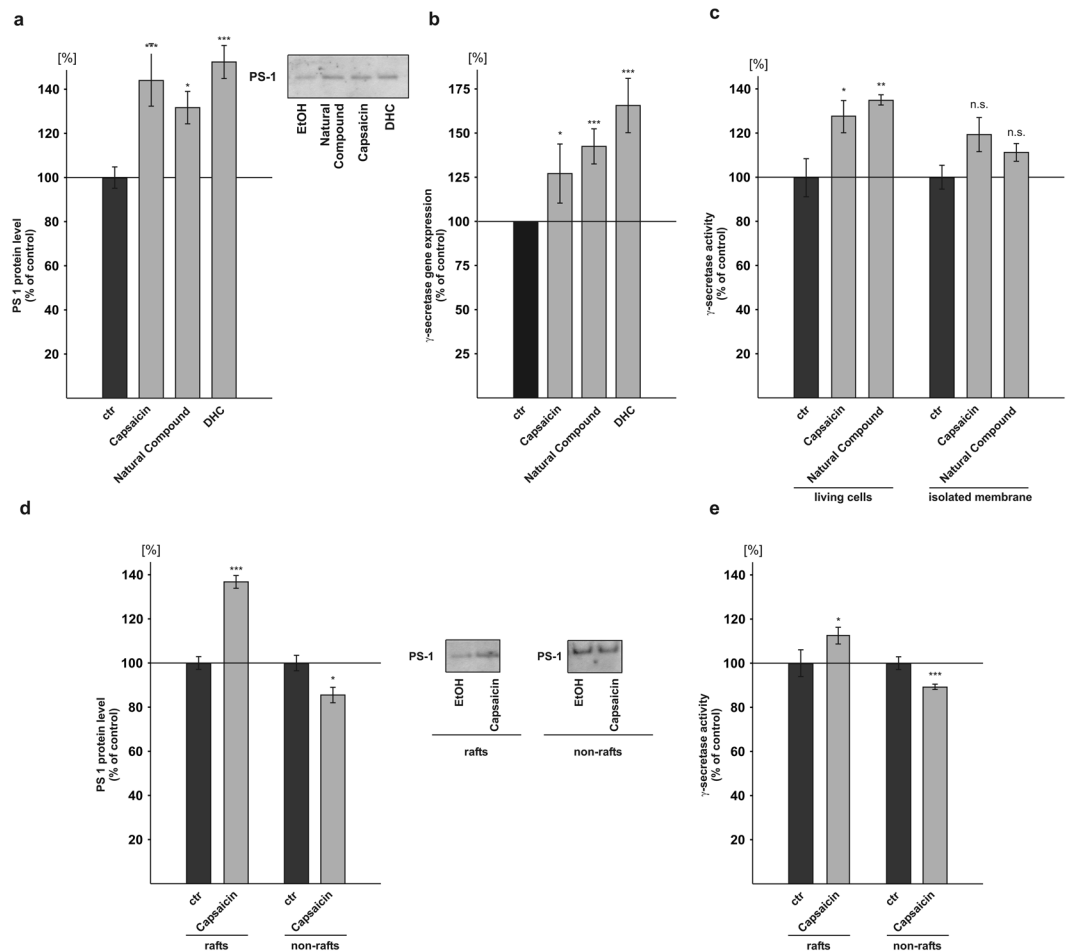


Figure 4. Effect of capsaicinoids on γ -secretase. **(a)** Levels of presenilin 1 (PS1) protein after treatment with capsaicin, natural compound and DHC on SH-SY5Y cells analyzed by WB ($n \geq 5$). **(b)** Gene expression analysis of γ -secretase complex components (*APH1a*, *NCSTN*, *PSEN1*, *PSEN2*, *PSENE1*) in capsaicinoid treated SH-SY5Y cells ($n = 4$). **(c)** γ -secretase activity in living cells ($n \geq 9$) and in isolated membranes ($n = 3$) of SH-SY5Y wt cells after capsaicin incubation. **(d)** Protein levels of PS1 in rafts and non-rafts after capsaicin incubation ($n = 3$). Representative, immunodetected PS1 signals are shown on the right. **(e)** γ -secretase activity in raft and non-raft fractions after capsaicin treatment ($n = 3$). Error bars represent the standard error of the mean. Asterisks show the statistical significance calculated by unpaired Student's t test or ANOVA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

	APH1a		NCSTN		PSEN1		PSEN2		PSENE1	
capsaicin	128 \pm 67%	0.6077	132 \pm 13%	0.0137*	128 \pm 5%	0.0002***	137 \pm 27%	0.1299	111 \pm 11%	0.2637
natural compound	156 \pm 19%	0.0055**	145 \pm 18%	0.0119*	155 \pm 19%	0.0073**	153 \pm 17%	0.0043**	103 \pm 3%	0.2783
dihydrocapsaicin	215 \pm 30%	0.0012**	190 \pm 18%	0.0002***	169 \pm 23%	0.0048**	156 \pm 21%	0.0096**	100 \pm 14%	0.9905

Table 1. mRNA levels of γ -secretase components. RT-PCR results for the single γ -secretase components including *APH1a*, *NCSTN* (nicastrin), *PSEN1* (PS1), *PSEN2* (PS2) and *PSENE1* (presenilin enhancer) in human neuroblastoma cells after incubation of capsaicinoids. Mean \pm standard error of the mean are listed on the left row and statistical significance calculated by unpaired Student's t test or ANOVA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) on the right row, respectively.

presence of capsaicin. The determination of γ -secretase activity in raft fractions revealed a statistically significant increase to 112.8% in presence of capsaicin, whereas γ -secretase activity was slightly but significantly decreased to 89.4% in non-raft fractions (γ -secretase activity rafts: 112.8% \pm 3.8%, $p = 0.034$; γ -secretase activity non-rafts: 89.4% \pm 1.2%, $p \leq 0.001$) (Fig. 4e).

Influence of capsaicinoids on cholesterol levels and cation channels. High cholesterol levels are implicated in the pathogenesis of AD^{79,80} and capsaicin has been shown to reduce cholesterol⁸¹. We therefore

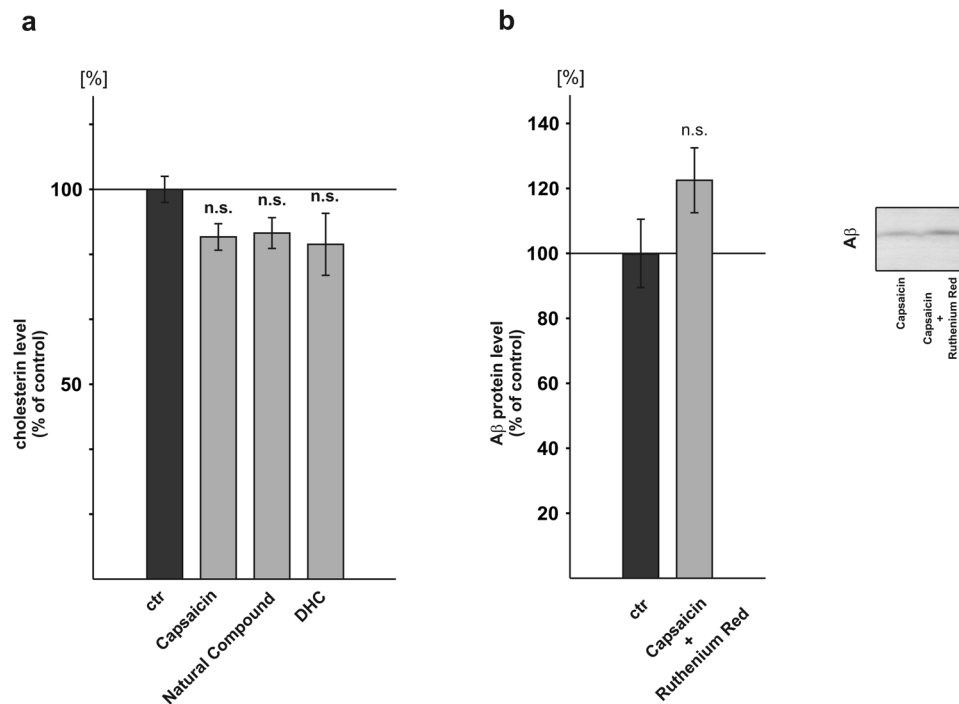


Figure 5. Influence of capsaicinoids on cholesterol levels and cation channels. **(a)** Cholesterol levels after capsaicinoid incubation in SH-SY5Y APP⁶⁹⁵ cells ($n \geq 3$). **(b)** Effect of Ruthenium Red and capsaicin on A β protein levels in SH-SY5Y APP⁶⁹⁵ cells ($n = 3$). Error bars represent the standard error of the mean. Asterisks show the statistical significance calculated by unpaired Student's t test or ANOVA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

analyzed whether capsaicin, the natural compound as well as DHC decrease cholesterol levels in our cell culture model. All analyzed compounds had a tendency to decrease cholesterol levels, however statistical analysis did not reach significance (capsaicin: $87.8\% \pm 1.0\%$, $p = 0.196$; natural compound: $88.8\% \pm 2.4\%$, $p = 0.280$; DHC: $85.9\% \pm 2.4\%$, $p = 0.195$) (Fig. 5a). However, one has to consider that in our experimental setting capsaicinoids were incubated for a short term (24 hours) and the effect of capsaicinoids on cholesterol might be more pronounced in the case of long-term incubation.

To investigate whether the observed effects of capsaicin on A β homeostasis are dependent on Ca²⁺ influx through cation channels, we incubated SH-SY5Y cells stably expressing APP⁶⁹⁵ in presence of capsaicin and Ruthenium Red. Ruthenium Red blocks cell membrane-located cation channels activated by capsaicin and blocks voltage-sensitive Ca²⁺ channels. If capsaicin would exert its effect via calcium influx, one would expect that by blocking these channels, secreted A β levels should be unchanged or decreased in presence of capsaicin and Ruthenium Red compared to cells only treated with capsaicin. However, in presence of capsaicin and Ruthenium Red secreted A β levels were not significantly elevated to $122.8\% (\pm 10.5\%, p = 0.199)$ compared to control cells incubated with capsaicin (Fig. 5b), indicating that the effect of capsaicin on A β homeostasis is independent of calcium influx in presence of capsaicin, which is in line with the finding that SH-SY5Y cells do not express TRPV1 channels⁸² and capsaicin therefore did not induce a calcium influx (Supplemental Fig. S2a). As a control, transient expression of TRPV1 in SH-SY5Y cells resulted in an increased basal as well as capsaicin induced calcium influx (Supplemental Fig. S2b).

Capsaicinoids decrease A β degradation by affecting IDE. Besides A β anabolism, total A β levels strongly depend on A β catabolism by A β degrading enzymes^{18,19}. To examine A β degradation in presence of capsaicinoids we incubated mouse neuroblastoma cells (N2a) with capsaicin, the natural compound or DHC in presence of human synthetic A β peptides. A β degradation was monitored by immunoblotting with antibody W02 recognizing human but not endogenous mouse A β . By utilizing the W02 antibody and adding A β peptides with the human A β sequence to a mouse cell line with neuronal properties, it is possible to measure the A β degradation independently of the endogenous A β production (see Supplemental Fig. S6).

In presence of capsaicin, remaining human synthetic A β peptides were significantly increased to 132.6% , indicating impaired A β catabolism. Similarly, for the other capsaicinoids, remaining A β peptides were significantly elevated to 135.7% in presence of the natural compound. An even stronger effect was observed for DHC revealing an increase to 146.8% (capsaicin: $132.6\% \pm 3.6\%$, $p \leq 0.001$; natural compound: $135.7\% \pm 6.2\%$, $p \leq 0.002$; DHC: $146.8\% \pm 8.4\%$, $p = 0.002$) (Fig. 6a). A β degradation in neuronal cell lines is mainly dependent on IDE and neprilysin⁸³. To further investigate whether the observed impaired A β degradation upon capsaicinoid treatment is mediated by IDE, we generated N2a cells knocked-down for IDE (see Supplemental Fig. S4), one of the main enzymes involved in A β degradation. Capsaicin only slightly increased remaining human A β peptides to 104.25% (capsaicin: $104.25\% \pm 1.3\%$, $p = 0.038$) in IDE knock-down cells (Fig. 6b) compared to the observed increase to

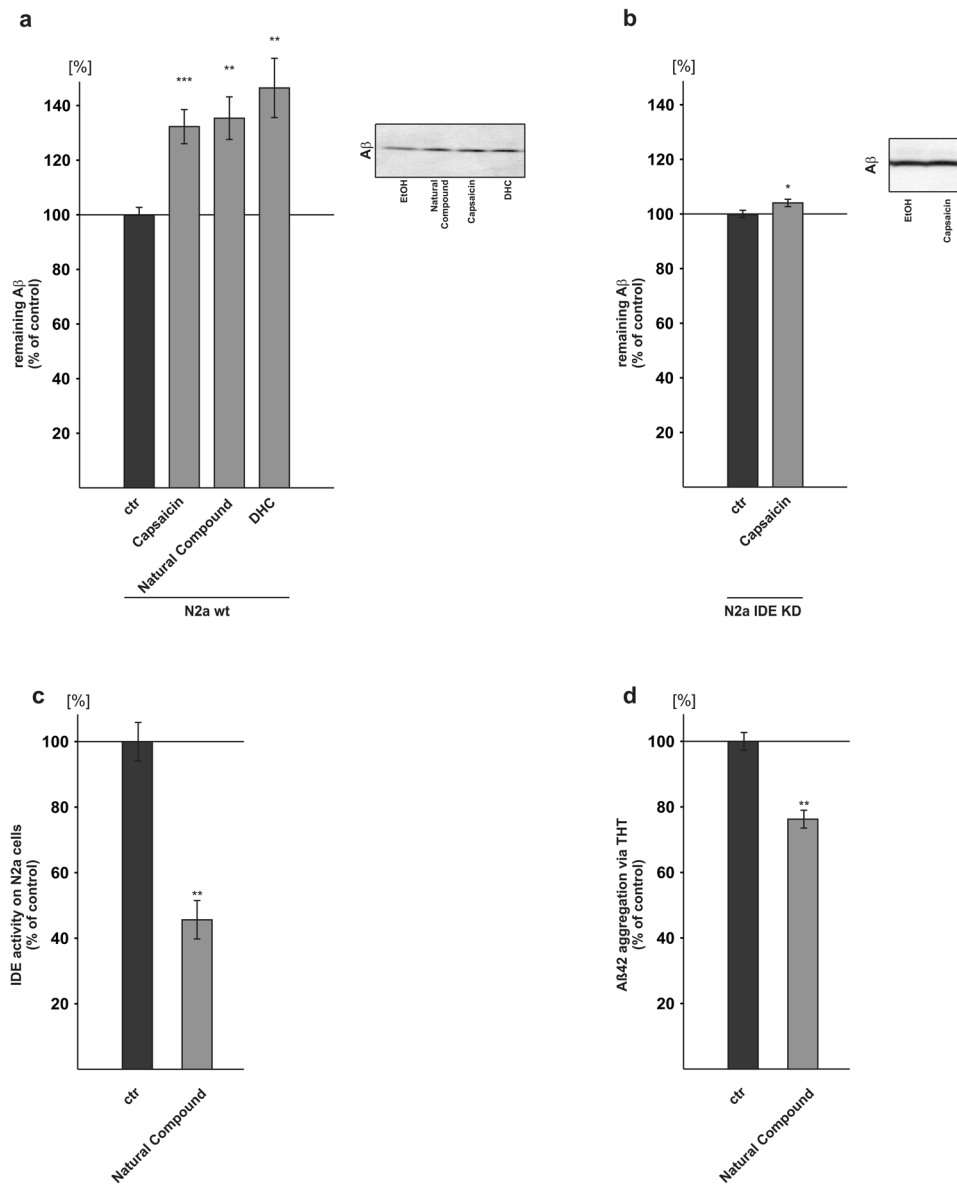


Figure 6. Effect of capsaicinoids on A β degradation and aggregation. **(a)** A β degradation was examined in capsaicinoid treated murine neuroblastoma cells (N2a) by WB ($n \geq 9$). **(b)** Influence of capsaicin on degradation of A β in N2a IDE knock-down cells ($n = 9$). **(c)** IDE activity assay in N2a cells after treatment with natural compound ($n = 6$). **(d)** THT-based A β aggregation assay ($n = 9$). Error bars represent the standard error of the mean. Asterisks show the statistical significance calculated by unpaired Student's t test or ANOVA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

132.6% in wild type N2a, indicating that IDE is affected by capsaicin. To confirm these results, an IDE activity assay was performed in presence of capsaicinoids revealing also a decreased IDE activity to 45.6% ($\pm 11.7\%$, $p = 0.008$) (Fig. 6c).

Effect of capsaicinoids on A β aggregation. Besides A β anabolism and A β catabolism, aggregation of A β peptides to small oligomers plays an important role in AD pathogenesis⁸⁴. Therefore, we monitored A β aggregation by the use of a fluorescent Thioflavin T-based assay. In presence of capsaicinoids (natural compound) A β aggregation was significantly decreased to 76.3% ($\pm 5.4\%$, $p = 0.010$) (Fig. 6d).

Discussion

AD is a multifactorial disease and its pathogenesis involves multiple pathological processes. According to the amyloid cascade hypothesis, A β accumulation in the brain of affected individuals causes synaptic dysfunction and neuronal cell death, leading to the well-characterized symptoms of the disease^{85,86}. AD has a long preclinical stage and A β generation starts years or decades before first symptoms appear to unambiguously manifest the disease. One of the current scientific approaches in respect to AD is to potentially delay the disease onset by bioactive

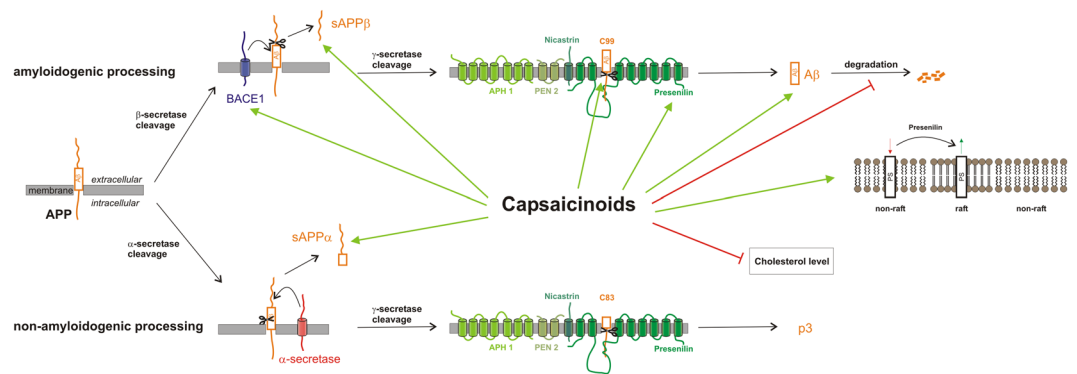


Figure 7. Mechanism of capsaicinoids action.

compounds lowering A β generation or A β aggregation. Several bioactive and/or dietary compounds have been described, which might be beneficial to treat or prevent AD, the most investigated being DHA^{32,87–89}. Curcumin, a natural polyphenol of *Curcuma* species, has a structure similar to capsaicin and is discussed to reduce A β levels and A β aggregation^{90–92}. Besides A β aggregation, oxidative stress and inflammation have been described as important factors to cause AD^{93,94}.

Capsaicin, the most prominent vanilloid in the fruits of *Capsicum* species, exerts pleiotropic pharmacological activities, including anti-inflammatory and anti-oxidant functions, which might be beneficial in respect to AD. However, so far the molecular mechanism how capsaicin affects APP processing and thus the generation of toxic A β species is not understood. In the present study we elucidated the effect of capsaicin, DHC and the natural compound consisting of several capsaicinoids on APP processing. All analyzed capsaicinoids significantly increased A β generation in the neuroblastoma cell line SH-SY5Y stably transfected with APP⁶⁹⁵ or the truncated construct C99, which only requires cleavage by γ -secretase to release A β . Elevated A β generation in presence of capsaicinoids was verified in the non-neuronal cell line COS7. The capsaicinoid induced elevation in total secreted A β levels are caused by an increase in gene transcription of β -secretase *BACE1* and the components of the γ -secretase complex, leading to elevated protein levels of BACE1 and PS1, the catalytically active component of the γ -secretase complex^{12,13}. Additionally, in presence of capsaicinoids PS1 shifts from non-raft to raft membrane microdomains, discussed to be involved in amyloidogenic APP processing⁹⁵. The observed elevation in gene transcription of the amyloidogenic secretases resulted in an increased enzyme activity of β - and γ -secretase detected in living SH-SY5Y cells incubated with capsaicinoids. Therefore, capsaicinoids exert their A β increasing effect rather indirectly, further substantiated by our finding that by the use of purified membranes no or only a marginal direct effect of capsaicinoids on β - or γ -secretase activity was observed. The increase of β - and γ -secretase activity was further confirmed in living HEK and N2a cells. No significant differences between capsaicin, dihydrocapsaicin and the natural compound were found in respect to A β generation, and the effect on the A β release seems to be independent of the single capsaicinoid structure. Besides increasing amyloidogenic APP processing, capsaicinoids decrease A β degradation by affecting IDE, also contributing to an elevation in total A β levels (Fig. 7). Our findings are in line with a previous study reporting that capsaicin promotes the amyloidogenic route of brain APP processing in capsaicin-treated rats⁴¹. In contrast, a recent study proposes that a capsaicin-rich diet may have protective effects on AD patients. In that study, a capsaicin-rich diet in humans, whose dietary habits regarding chili pepper consumption were collected using a Food Frequency Questionnaire, reduced serum A β 40 levels as well as total serum A β levels, and revealed positive effects on cognitive functions. Serum A β 42 levels were unaffected⁴⁰. However, as discussed by the authors, blood A β has limitations as a marker to reflect AD related pathological changes. The analysis of cerebrospinal fluid would be more favorable. Nevertheless, further studies addressing cognitive impairment in mice proposed beneficial effects of capsaicin in mice. Chen *et al.* reported that daily treatment with capsaicin (1 mg/kg, i.p.) improved spatial learning as well as memory synaptic function in C57Bl/6 mice microinjected with 100 μ M A β 42⁹⁶. Treatment with the TRPV1 antagonist capsaizepine (1 mg/kg, i.p.) showed no effects on cognitive and synaptic function in these mice, suggesting that the above mentioned effect may be mediated through the activity of TRPV1 channels. Similarly, capsaicin has been shown to ameliorate cognitive impairment and tau hyperphosphorylation provoked by cold water stress in rats³⁹. Also, reduced hyperphosphorylation of tau proteins has been reported in the hippocampus of type2 diabetes rat, known as risk factor for the development of AD, fed with a capsaicin-rich diet³⁷. Interestingly, the capsaicin diet did not reduce tau hyperphosphorylation in healthy rats not affected by type2 diabetes, indicating that dietary capsaicin might prevent AD in individuals affected by type2 diabetes. Capsaicin, present as bioactive compound in Kimchi, a traditional Korean fermented vegetable, also had a tendency to decrease phosphorylated tau proteins in the brain of A β -injected mice⁹⁷. In this study protein expression of APP and BACE1, elevated by A β injection, also tended to decrease by oral administration of 10 mg capsaicin. Furthermore, daily oral administration of bioactive compounds in Kimchi, including capsaicin (10 mg/kg), improved cognitive and memory functions in mice injected with A β 25–35⁹⁸. Recently it has been shown that TRPV1 activation by capsaicin rescued A β -induced degradation of hippocampal gamma oscillations, a cognition-relevant EEG pattern, by reversing desynchronization of AP firing in CA3 pyramidal cells and by reversing the shift in the excitatory/inhibitory current balance⁹⁹. This

reversing effect was not detectable in TRPV1 knock-out mice or in presence of the antagonist capsazepine, clearly pointing towards a role of TRPV in mediating these effects.

Although these studies propose beneficial effects in tests of cognition and show decreased tau hyperphosphorylation associated with AD, our results indicate that capsaicinoids as bioactive compounds have additional effects on APP processing and A β homeostasis. In the present study capsaicinoids significantly increased total A β levels by increasing amyloidogenic APP processing and by reducing A β degradation and might therefore not be unconditionally recommended as a dietary supplement to prevent AD or to delay the disease onset. On the other hand we also found beneficial effects of capsaicinoids in respect to AD as we could show that capsaicinoids reduced A β aggregation. Interestingly, vanillin and curcumin which are structurally related to capsaicinoids have been shown to decrease A β aggregation¹⁰⁰. Our results imply that potentially a TRPV1 agonist that does not affect APP processing might be preferential, which has to be proven in further studies.

Given the multifactorial nature of the disease including various pathological processes, it is difficult to judge whether a compound might be beneficial or detrimental when only one pathological aspect in AD, e.g. A β generation or tau hyperphosphorylation, is investigated.

As a limitation of our study, it has to be pointed out that this is a sole *in vitro* study aiming to elucidate the molecular effect of capsaicinoids on A β homeostasis. Our study investigates the effect of capsaicinoids on SH-SY5Y cells in a short time period and in the absence of functional TRPV1 channels. The concentration used in this study is supraphysiological. It has to be highlighted that capsaicinoids are one of the most frequently consumed spices in humans. A (life)-long exposure of a continuous but low concentration is hard to simulate in cell culture. Therefore, we decided to use 24 hours long exposure of a higher concentration to investigate if there is in principle an impact of capsaicinoids on A β homeostasis or the amyloidogenic pathway. The rationale for using 10 μ M capsaicinoids was that at this concentration no cytotoxicity was observed and other studies have used a similar or higher concentration range to determine the potential effect of capsaicinoids on different cellular pathways^{101–106}.

Moreover, dietary habits such as persistent consumption of spicy food might therefore have long term effects, potentially mediated by TRPV1, which were not reflected in this study. E.g. one might assume that the cholesterol-lowering effect would be more pronounced *in vivo*, when capsaicin is consumed over a long period by diet.

As the experiments were performed in SH-SY5Y cells and as these cells do not express TRPV1⁸², which was experimentally verified by us, our results show that the observed increase in A β levels is likely independent of a change in calcium homeostasis. However, we do not rule out that in other cells highly expressing the TRPV1 channels, potential changes in calcium homeostasis induced by capsaicin might also interfere with the A β homeostasis. However, besides increasing total A β levels, in our study capsaicinoids revealed also an increase in sAPP α levels. The sAPP α fragment has neurotrophic and neuroprotective functions and is reported to be decreased in some cases of AD^{107,108}.

In summary, capsaicin has been shown in literature to be beneficial in respect to tau pathology in AD and by reducing ROS and inflammation. Here we show additional and potential undesirable effects of capsaicin on APP processing and A β homeostasis, emphasizing the need for further studies before a clear advice of capsaicin in respect to AD prevention and treatment can be given.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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T.B., A.A.L., D.J., C.S., J.M., V.J.H. and A.M.M. performed the experiments; T.B., A.A.L., D.J., H.S.G., M.O.W.G., B.A.N. and T.H. wrote the manuscript; M.O.W.G. and B.A.N. designed the study.

Competing interests

The authors declare no competing interests.

Additional information

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