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# Impaired Geranylgeranyltransferase-I Regulation Reduces Membrane-Associated Rho-Protein Levels in Aged Mouse Brain

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## Abstract

Synaptic impairment rather than neuronal loss may be the leading cause of cognitive dysfunction in brain aging. Certain small Rho-GTPases are involved in synaptic plasticity, and their dysfunction is associated with brain aging and neurodegeneration. Rho-GTPases undergo prenylation by attachment of geranylgeranylpyrophosphate (GGPP) catalyzed by GGTase-I. We examined age-related changes in the abundance of Rho- and Rab proteins in membrane and cytosolic fractions as well as of GGTase-I in brain tissue of 3 and 23 mo old C57BL/6 mice. We report a shift in the cellular localization of Rho-GTPases towards reduced levels of membrane-associated and enhanced cytosolic levels of those proteins in aged mouse brain as compared with younger mice. The age-related reduction of membrane-associated Rho-proteins was associated with a reduction in GGTase-I levels that regulates binding of GGPP to Rho-GTPases. Proteins prenylated by GGTase-II were not reduced in aged brain indicating a specific targeting of GGTase-I in the aged brain. Inhibition of GGTase-I *in vitro* modeled the effects of aging we observed in vivo. We demonstrate for the first time a decrease of membrane-associated Rho proteins in aged brain in association with down-regulation of GGTase-I. This down-regulation could be one of the mechanisms causing age-related weakening of synaptic plasticity.

### Keywords

Isoprenoid; Rho protein; brain; aging; small GTPases; synaptic markers

## Introduction

Synaptic impairment rather than neuronal loss may be the leading cause of cognitive dysfunction in brain aging (Grillo *et al.* 2013, Burke & Barnes 2006b, Morrison & Baxter

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Conflicts of interest: Sazetidine-A was developed by KJK and YX and Georgetown University currently holds the patent on sazetidine-A.
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2012). Age-related synaptic dysfunction is most likely due to deterioration of synaptic contacts between axonal buttons and dendritic spines (Mostany *et al.* 2013, Hof & Morrison 2004). Immunoreactivity of synaptic markers such as synaptophysin and GAP43 decreased in an age-dependent manner in human and rodent brains (Saito *et al.* 1994, Casoli *et al.* 1996, Keleshian *et al.* 2013). Decreases in spine density, which correlates with functional impairment (Peters *et al.* 2008) have been reported in aging rodents (Wallace *et al.* 2007, Bloss *et al.* 2013), non-human primates (Page *et al.* 2002), and humans (Anderson & Rutledge 1996, Mostany et al. 2013). Recent *in vivo* two-photon imaging revealed alterations in the size and stability of spines and boutons during normal brain aging (Grillo et al. 2013, Mostany et al. 2013).

The small GTPases Rac1, RhoA and Cdc42 have emerged as crucial regulators of neuronal morphogenesis supporting synaptic plasticity (Gonzalez-Billault *et al.* 2012). The majority of small Rho-GTPases are prenylated by GGPP involving geranylgeranyltransferase-I (GGTase-I), which catalyzes the covalent attachment of geranylgeranyl moiety via thioether linkage to the CAAX-motif of those proteins (Fig 1). The functional roles of brain prenylated proteins are well studied, which is in contrast to knowledge of the prenylation process. It has only been recently reported that the two isoprenoids, which prenylate proteins, farnesyl pyrophosphate (FPP) and GGPP were quantified reliably in human and murine brain tissue (Hooff *et al.* 2008, Hooff *et al.* 2010a). We reported that GGPP and FPP levels were significantly elevated in brain tissue of aged mice and AD patients when compared with younger mice and age-matched controls, respectively (Eckert *et al.* 2009, Hooff *et al.* 2012). Reducing GGPP levels decreases abundance of prenylated proteins in membrane fractions of primary neurons (Ostrowski *et al.* 2007, Rilling *et al.* 1993). Prenylation of small GTPases enhances insertion of the proteins into cellular membranes (Garcia-Mata *et al.* 2011), which is required for their active state (Samuel & Hynds 2010). Therefore, we tested the overall hypothesis that the abundance of membrane-associated small GTPases is reduced in aged brain. Moreover, we investigated if the increase in GGPP levels that has been detected in aged brain could be due to up-regulation of this key isoprenoid or alternatively a consequence of impaired function of GGTase-I and II.

## Materials & Methods

### Chemicals and Reagents

GGTase-I was obtained from Jena Bioscience (Jena, Germany) and D*-GCVLL (dansyl gly-cys-val-leu-leu) from Calbiochem (Darmstadt, Germany). Ammonium hydroxide solution 28–30% was purchased from Alfa Aesar (Karlsruhe, Germany), the phosphatase inhibitors Halt® and Phosstop® from Thermo-Fisher/Piercenet (Bonn, Germany) and Roche Diagnostics GmbH (Mannheim, Germany) and the GGTase-I inhibitor GGTI-2133 from Sigma Aldrich (Schnelldorf, Germany). All solvents were of analytical grade or higher quality. Acetonitrile was obtained from Carl Roth GmbH (Karlsruhe, Germany), 1-butanol, n-hexane, 2-propanol, methanol, acetone, ammonium acetate and assay buffer compounds: Tris–HCl, $MgCl_2$, $ZnCl_2$ and $Na_2CO_3$ were obtained from Merck (Darmstadt, Germany). GGPP, octyl- -Dglucopyranoside and dithiothreitol were from Sigma-Aldrich (Schnelldorf, Germany). Millipore water was used for all solutions (Schwalbach, Germany).

### Animals

Male C57BL/6 mice (3 and 23 months of age) were obtained from Janvier (St. Berthevin Cedex, France). The mice were maintained on a 12-h dark–light cycle with pelleted food and tap water *ad libitum*. In the design of the experiments the ARRIVE guidelines were followed. All experiments were carried out by individuals with appropriate training and appropriate experience in accordance with the European Communities Council Directive (86/609/EEC) and the ARRIVE guidelines.

### Brain tissue preparation

Brains were dissected into two hemispheres (without brain stem and cerebellum), snap frozen in liquid nitrogen and stored at −80°C until use. For the mRNA analysis, the frontal cortices of the second hemispheres were used, while all other experiments were performed using the entire hemisphere (without brain stem and cerebellum).

### Protein analysis

Protein levels were measured using the BCA Protein Assay Kit from Thermo-Scientific/ Pierce (Bonn, Germany). Samples were measured in triplicates.

### Membrane isolation

Brain membrane and cytosolic fractions were isolated according to Ma et al., 2008 (Ma *et al.* 2008). Briefly, tissue samples were sequentially processed by homogenization and ultracentrifugation (100,000 g for 20 min) to obtain supernatants (TBS, soluble-cytosol fraction). Pellets were then sonicated in lysis buffer and again centrifuged to obtain lysis extract supernatants (membrane-cytoskeletal extract).

Cell membrane fractions of human SH-SY5Y neuroblastoma cells (cells were generously provided by Dr. Bernd Fiebich, University of Freiburg) and were isolated according to Ostrowski et al., 2007 (Ostrowski et al. 2007). Briefly, cells were lysed by incubation in relaxation buffer on ice for 15 min followed by a 10-s sonication. Cells were cleared by centrifugation at 500 *g* for 5 min at 4 °C. The resulting supernatant was centrifuged for 1 h at 110,000 *g* at 4 °C in a Beckman-Coulter ultracentrifuge (70.1TI rotor). The resulting supernatant was removed (cytosolic fraction), and the membrane pellet was then resuspended in relaxation buffer (membrane fraction).

### Cell culture

Human SH-SY5Y neuroblastoma cells were cultured in MEM medium (Sigma Aldrich, Schnellendorf, Germany) as previously described (Hooff *et al.* 2010b) at 37°C and 5% $CO_2$. For incubation with the GGTase-I inhibitor GGTI-2133 cells were kept in serum-free OptiMEM medium supplemented only with penicillin/streptomycin. Cells were incubated for 48 hr and treated twice at 0 hr and 24 hrs with the GGTase-I inhibitor GGTI-2133 dissolved in DMSO. After harvesting, cells were centrifuged and washed twice with PBS containing Complete® protease-inhibitor cocktail (Roche, Mannheim Germany). Pellets were resuspended and homogenized in 50 mM TrisHCl (pH = 7.4).

### RNA Isolation, Reverse Transcription, Primer Design, and qRT-PCR

RNA was isolated from brain frontal cortex using the Trizol method and purified with Invitrogen's ChargeSwitch®. Total RNA Cell Kit procedures were used according to the manufacturer's instructions (for details please refer to the supplementary method description).

### Western blot analysis

For specific protein determination, samples were prepared by diluting (in total cell or brain tissue homogenate: 10 μg for GAP43, synaptophysin, GGTase-I , small GTPases (Rac1, RhoA, Cdc42, Rab3A and Rab11B); in membrane and cytosolic preparations: 80 μg for Rac1, Cdc42 and Rab3A, 40 μg for RhoA and Rab11B, 5 μg for RhoGDI and RabGDI ) protein with the reducing agent (10x) and NuPAGE LDS Sample buffer (4x). After denaturation for 10 min at 95°C, the samples were electrophoretically separated on a 4-12% NuPAGE Bis/Tris gel (Invitrogen, Germany) for 40 min at 190 V and then transferred on a PVDF membrane for 90 min at 30 V and blocked with 7.5% non fat dried milk in Millipore water for 30 min. Membranes were incubated with primary antibodies (for details please refer to the supplementary method description). Band analysis was performed using BioRad's Quantity One Software.

### GGTase activity Assay

Relative GGTase activity was measured in brain tissue according to Goosens et al. 2005 (Goossens *et al.* 2005). Briefly, brain tissue was homogenized in lysis buffer. The homogenate was centrifuged at 10.000 g for 30 min at 4°C. The resulting supernatant was then centrifuged at 100.000 g for 60 min at 4°C and the resulting supernatant (cytosolic fraction) was aliquoted and stored at −80°C until use. The reaction mixture for the activity Assay was mixed with the cytosolic fraction. The activity was determined by measuring the value of fluorescence (excitation 340 nm, emission 505 nm) using a Bowman II Aminco spectrofluorometer (SLM Aminco, Urbana, USA). Cuvette temperature was 37°C.

### Isoprenoid analysis

Determination of GGPP levels in SH-SY5Y cells and mouse brain homogenates was performed using a validated HPLC-FD method as previously described (Hooff et al. 2010b).

### Statistics

All data are expressed as means ± standard error of the mean (SEM) unless stated otherwise. For direct comparison of differences between two and three groups, student's t-test and one-way ANOVA followed by Tukey's post-test were calculated, respectively. All calculations were performed with GraphPad Prism version 5.00 for Mac, GraphPad software, San Diego, USA.

## Results

### Brain levels and cellular distribution of Rho- and Rab GTPases in young and old mice

The Rho family of proteins has a major role in neuronal homeostasis and as signaling mediators in glia cells (Feltri *et al.* 2008, Hooff *et al.* 2010c). For those reasons, we focused on the Rho family proteins. Protein abundance of Rho- GTPases in membrane and cytosolic fractions as well as in total homogenates were determined in brain lysates from 3 and 23 months old C57BL/6J mice.

Membrane-associated Rac1 was reduced significantly by about 30% in mice 23 mo of age as compared with 3 mo old mice (Fig. 2a). The reduction of membrane-associated Rac1 in aged brain was not due to a decrease in the total amount of Rac1 protein (Fig. 2b). In a separate set of experiments we found that cytosolic Rac1 protein levels were elevated in aged mice as compared with younger mice (Fig. 3a). Similar results were observed for RhoA and Cdc42 (Fig. 2 c-f & 3b,c). Membrane-associated Rho proteins are reduced in aged brains. Cytosolic GDP-dissociation inhibitor proteins (GDI) keep GTPases in the inactive GDP-bound state by blocking nucleotide exchange and thereby regulating membrane association of GTPases (Cherfils & Zeghouf 2013, DerMardirossian & Bokoch 2005). GDI binding of Rho proteins could be one explanation for the reduction in membrane GTPases in aged mice. Levels of RhoGDI- which specifically binds to Rac1, RhoA and Cdc42 (Olofsson 1999, Wennerberg & Der 2004, Pfeffer & Aivazian 2004) and RabGDI- which is enriched in brain (Alory & Balch 2001) were unchanged in our samples (Fig. 4a & b).

Prenylation of Rho proteins requires the activity of the transferase GGTase-I (Hooff et al. 2010c). GGTase-II prenylates members of the Rab protein family (Fig. 1). We next examined if the age-related reduction in membrane-associated Rho-protein was specific to proteins acted on by GGTase-I or if similar effects would be seen in proteins prenylated by GGTase-II. Protein levels of Rab11B and Rab3A which are abundant in the central nervous system (Stenmark & Olkkonen 2001, Kelly *et al.* 2012) were determined in mice 3 and 23 mo old mice. Data in Figure 3&5 indicate a mixed message regarding prenylation of Rab-proteins. Age differences in membrane-associated and cytosolic Rab11B levels were not observed (Fig 5a & 3d,). Membrane-associated Rab3A levels however were increased (Fig. 5 c) and reduced in the cytosolic fractions (Fig. 3e) in brain tissue of 23 mo old mice. Total Rab11B- and Rab3A protein levels (Fig. 5 b,d) were similar for the two age groups.

### GGTase activity, GGTase-I protein and gene expression levels are reduced in aged mouse brain

Abundance of membrane-associated Rac1, RhoA and Cdc42 were reduced in aged brain but not Rab-proteins (Figs 2&5). Both protein families require GGPP for prenylation. However, a deficiency in GGPP levels cannot account for the age-related reduction in prenylated Rho-proteins. Brain GGPP levels were actually higher in aged as compared to younger mice (Fig. 6a). GGTase-I is a heterodimer consisting of an subunit which is identical to the subunit of farnesyl transferase and a separate subunit which regulates binding of GGPP to Rac1, RhoA, Cdc42 and other Rho proteins (Casey 1996; Taylor et al., 2003). Figure 6b shows that

GGTase-I protein levels were significantly lower of about 25% in brain tissue of aged than young mice. This reduction corresponds to the diminished levels of membrane bound Rho GTPases which are in the same range (Fig. 2). The reduction in GGTase-I protein levels was associated with a decrease in GGTase-I transcription. Data in Figure 6c show that there was approximately an 80% decrease in GGTase-I mRNA expression levels in the aged brain in contrast to the younger group. Reduced GGTase-I transcription and protein levels at least contributed to the observed age-related reduction in relative GGTase activity (Fig. 6d).

### Directly inhibiting GGTase-I in SH-SY5Y cells mimics the effects of aging

Relative GGTase activity, GGTase-I protein levels and gene expression were significantly lower in brain tissue of aged as compared with younger mice as discussed above. Those changes in GGTase-I were associated with a shift of protein localization towards reduced abundance of prenylated membrane-associated Rac1, RhoA and Cdc42 in aged brain. To further examine the role of GGTase-I on Rac1 prenylation, SH-SY5Y cells were incubated with the specific GGTase-I inhibitor GGTI-2133 (Fig. 1). The results of these *in vitro* experiments are similar to what we observed in brain of aged mice (Fig. 2-4): Figure 7 shows that inhibition of GGTase-I induces a shift of protein localization towards reduced abundance of prenylated membrane-associated Rac-1 (Fig. 7a) and enhanced protein levels in the cytosol (Fig. 7b). Total Rac-1 levels were unchanged (Fig. 7c). Cytosolic RhoGDIα (Fig. 7d) and RabGDIα (Fig. 7e) protein levels were similar in GGTI-2133 treated and control SH-SY5Y cells. Prenylation of Rab-proteins was not affected by inhibition of GGTase-I (data not shown). Figure 6f shows that inhibition of GGTase-I by GGTI-2133 significantly increased GGPP levels, which parallel the increase observed in the aged brain (Fig. 7a).

### Increasing age and direct inhibition of GGTase-I are associated with a reduction of synaptic markers

Synaptophysin, a glycoprotein component of presynaptic vesicle membranes, and growth-associated protein GAP43, a component of growth cone membranes, are exclusively expressed and distributed in synapses. Those proteins are commonly used as synaptic markers (Jahn *et al.* 1985, Wiedenmann & Franke 1985). Data in Figure 8c & d show that both synaptophysin and GAP43 protein levels were significantly lower in aged mouse brain as compared with younger mice. Directly inhibiting GGTase-I activity by using GGTI-2133 in SH-SY5Y cells significantly decreased synaptophysin and GAP43 protein levels (Fig. 8a&b). Those *in vitro* results are similar to what we observed in aged brain and raises albeit speculative notion that GGTase-I deficiency (Fig. 6 b-d) contributes to synaptic loss in brain of aged mice.

## Discussion

Aging is associated with cognitive decline, which is related to synaptic plasticity (Burke & Barnes 2006a, Burke & Barnes 2010). Much attention has focused on changes in dendritic branching and spine density underlying age-related reduction in synaptic plasticity but mechanisms for those changes are not well understood (Burke & Barnes 2006a, Burke & Barnes 2010). The actin cytoskeleton plays a critical and essential role in controlling

development and maintenance of spines and synapses (Tolias *et al.* 2011). Organization and function of the actin cytoskeleton is dependent on the Rho family of proteins. Developmental studies and studies on specific forms of mental retardation have demonstrated the critical importance of Rho-proteins such as Rac1, RhoA and Cdc42 in governing synapse development and plasticity (Tolias et al. 2011, Chen *et al.* 2012, Newey *et al.* 2005, Bongmba *et al.* 2011). Normal functioning of these proteins requires the attachment of the 20-carbon GGPP on the cysteine residue of a carboxy terminal CAAX motif. Prenylated proteins can undergo up to three more post-translational-modifications which all increase protein hydrophobicity and facilitate membrane association which is required for their active state (reviewed in (Hooff *et al.* 2011, Boulter *et al.* 2012, McTaggart 2006, Samuel & Hynds 2010)). We found a shift in the cellular localization of Rho-GTPases towards reduced levels in membranes and enhanced levels in cytosolic fractions in aged mouse brain as compared with younger mice. Thus, our findings of reduced membrane-associated Rho proteins might have functional consequences for the aging brain.

The age-related reduction of membrane-associated Rho-proteins was associated with a reduction in protein and mRNA levels of GGTase-I, a subunit of GGTase-I that regulates binding of GGPP to Rho-GTPases. Inhibition of GGTase-I *in vitro* mimicked the changes we observed in the brain of aged mice including reduced abundance of synaptic markers. The reduction in membrane-associated Rho-proteins was specific for those proteins prenylated by the transferase GGTase-I but not Rab-proteins prenylated by GGTase-II. A consequence of GGTase-I down-regulation in aged brain may be a contributing factor to synaptic impairment that occurs with increasing age. LTP was impaired in Rac1-deficient mice that also showed deficits in spatial learning and fewer neuronal synapses (Haditsch *et al.* 2009). In contrast, activation of brain Rho-GTPases improved learning and memory in C57BL/6 mice (Diana *et al.* 2007).

Data in the present study indicate that down-regulation of GGTase-I reduces membrane binding of Rho proteins in the aged brain. However, an alternative mechanism for reduction of prenylated Rho proteins involves the guanine dissociation inhibitors (GDIs) which influence GTPase function and localization (Cherfils & Zeghouf 2013). In the cytosol, GDIs keep GTPases in the inactive GDP-bound state by blocking nucleotide exchange and thereby regulating membrane association of GTPases (DerMardirossian & Bokoch 2005). We show that protein levels of RhoGDI- and RabGDI- were similar in cytosolic fractions isolated from brains of young and aged mice indicating that age-related changes in Rho- and Rab protein levels are not due to GDI. Although not in the focus of this paper

Since we did not investigated prenylated proteins in the cytosol future experiments should clarify if during aging there is a bona fide increase of unprenylated Rho protein levels especially since recent findings indicate that the largest proportion of prenylated Rho family members is found in the cytosol acting as a reservoir of Rho proteins that can be rapidly translocated to membranes (Boulter *et al.* 2010). These investigations should also take into account that kinases such as PKA or Src act on either GDIs or Rho-GTPases themselves to enhance or decrease GTPase-GDI binding affinity and therefore regulate the size of Rho-protein pool in the cytosol (Boulter et al. 2012).

Our data identified a critical role of GGTase-I in brain aging. GGPP is a substrate of GGTase-I, which is a cytosolic protein consisting of an alpha and a beta-subunit (Lane & Beese 2006). The GGTase-I -subunit is identical to the FTase -subunit and the GGTase-I -subunit directs protein substrate selectivity. GGTase-I is highly enriched in brain, and there is increasing interest in the important role of GGTase-I in dendritic development (Wu *et al.* 2010, Gonzalez-Billault et al. 2012). Studies on GGTase-I in brain have focused on its function early in the lifespan. Suppression of GGTase-I in hippocampal neurons reduced dendritic arborization while overexpression of GGTase-I had the opposite effect (Zhou *et al.* 2008a). In the same publication, it has been shown that the beneficial effects of GGTase-I were inhibited in cells overexpressing Rac1 protein with the prenylation site deleted or mutated. Both cognitive function and LTP are reduced with increasing age (Burke & Barnes 2006a), which may be due in part to defective regulation of GGTase-I. Manipulating the levels of isoprenoids and protein prenylation modulates synaptic plasticity and cognitive function in animal models (Mans *et al.* 2010, Mans *et al.* 2012, Li *et al.* 2006, Ye & Carew 2010, Cheng *et al.* 2013, Costa *et al.* 2002). A recent study demonstrated that GGTase-I mediates synaptogenesis through BDNF-induced Rac1-activation (Li *et al.* 2013) which is directly related to our data. Li et al. showed that inhibition of GGTase-I reduced the levels of the pre-synaptic marker Synapsin1 and the post-synaptic density protein 95 (Li et al. 2013). Since age-related reductions in synaptophysin and GAP43 levels have been reported in aged rodent brain (Saito et al. 1994, Casoli et al. 1996, Keleshian et al. 2013) we focused on those pre-synaptic markers. We confirmed that synaptophysin and GAP43 protein levels are reduced in brains of aged mice. Moreover, inhibition of GGTase-I *in vitro* decreased synaptophysin and GAP43 levels as we observed in aged mouse brain, further confirming the critical role of GGTase-I in brain aging. Our data may also have impact on glial related functions of small GTPases, such as directional migration of oligodendrocytes precursor cells (Biname *et al.* 2013) or inhibition of adhesion and migration of microglia (Yan *et al.* 2012).

Rab3A is a protein associated with the membrane of synaptic vesicles and is involved in the control of the targeting or docking of these vesicles at the presynaptic membrane for the release of neurotransmitters (Stettler *et al.* 1994). Rab3A gene expression significantly decreases with aging and in Alzheimer's disease (Saetre *et al.* 2011). We report on enhanced Rab3A protein levels in membrane preparations isolated from aged brain. However, if this novel finding contributes to age-related synaptic dysfunction needs further investigation.

GGTase-I inhibition significantly increased GGPP levels in SH-SY5Y cells. These *in vitro* results simulate what we observed in brain tissue of aged mice and may provide an explanation for the increase in GGPP levels seen in brains of aged mice (Hooff et al. 2012). The age-related reduction in relative GGTase activity causes an abnormal accumulation of GGPP. The conventional view is that GGPP is primarily involved in protein prenylation. However, there is limited evidence that GGPP may have effects independent of protein prenylation, such as inhibition of choline phosphotransferase (Miquel *et al.* 1998) and stimulation of -secretases (Zhou *et al.* 2008b).

Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and it is a risk factor for Alzheimer disease (AD) (Lopez-Otin *et al.* 2013). We

reported enhanced levels of GGPP and FPP in brains of aged mice and in post-mortem brain tissue from AD patients (Eckert et al. 2009, Hooff et al. 2012). Taken together these and other data indicate that age related changes in protein prenylation and isoprenoid levels might have an impact on AD (Li *et al.* 2012). However, a recent report on heterozygous deletion of FTase and GGTase-I showed reduced levels of amyloid beta-protein and neuroinflammation in a mouse model of AD (Cheng et al. 2013) which can be interpreted as neuroprotective. The functional roles of prenylated proteins and isoprenoids may differ in normal aging as compared with AD.

In conclusion, we report the novel finding that GGTase-I protein and gene expression levels were significantly lower in aged mouse brain as compared with younger mice. Age-related down-regulation of GGTase-I was associated with reduced abundance of membrane-associated Rac1, RhoA and Cdc42. Direct inhibition of GGTase-I *in vitro* mimicked effects observed in aged mouse brain. Thus, GGTase-I down-regulation could be one of the mechanisms contributing to impaired synaptic plasticity that occurs in aged brain.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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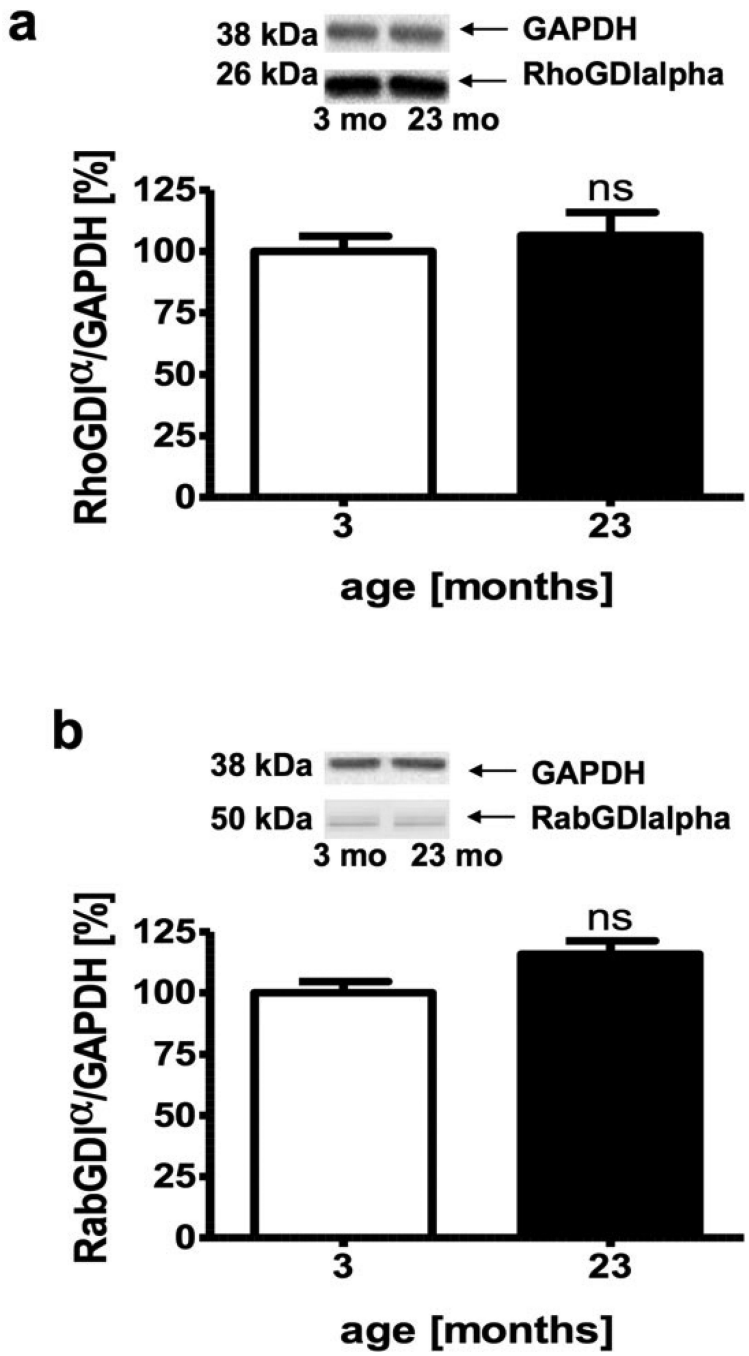


**Figure 1. Abbreviated mevalonate/isoprenoid/cholesterol pathway**

The mevalonate pathway is a crucial metabolic pathway in eukaryotic cells for which mevalonate is the precursor of several compounds including the isoprenoids farnesyl- (FPP), geranylgeranyl-pyrophosphate (GGPP) and cholesterol. The synthesis of GGPP and FPP is catalyzed by farnesylpyrophosphate synthase (FDPS) and geranylgeranylpyrophosphate synthase (GGPPS), respectively. GGPP is substrate for post-translational geranylgeranylation of small GTPases by geranylgeranyltransferases (GGTase). GGTase-I prenylates Rho-GTPases (Rac-1, RhoA, Cdc42) and GGTase-II prenylates Rab-GTPases (Rab3A, Rab11B). Geranylgeranylation of these proteins (-GG) is critical for membrane localization and optimal function. GGTI-2133 is

a specific inhibitor of GGTase-I. Aging reduces the relative GGTas-I activity leading to reduced levels of prenylated Rho-proteins.

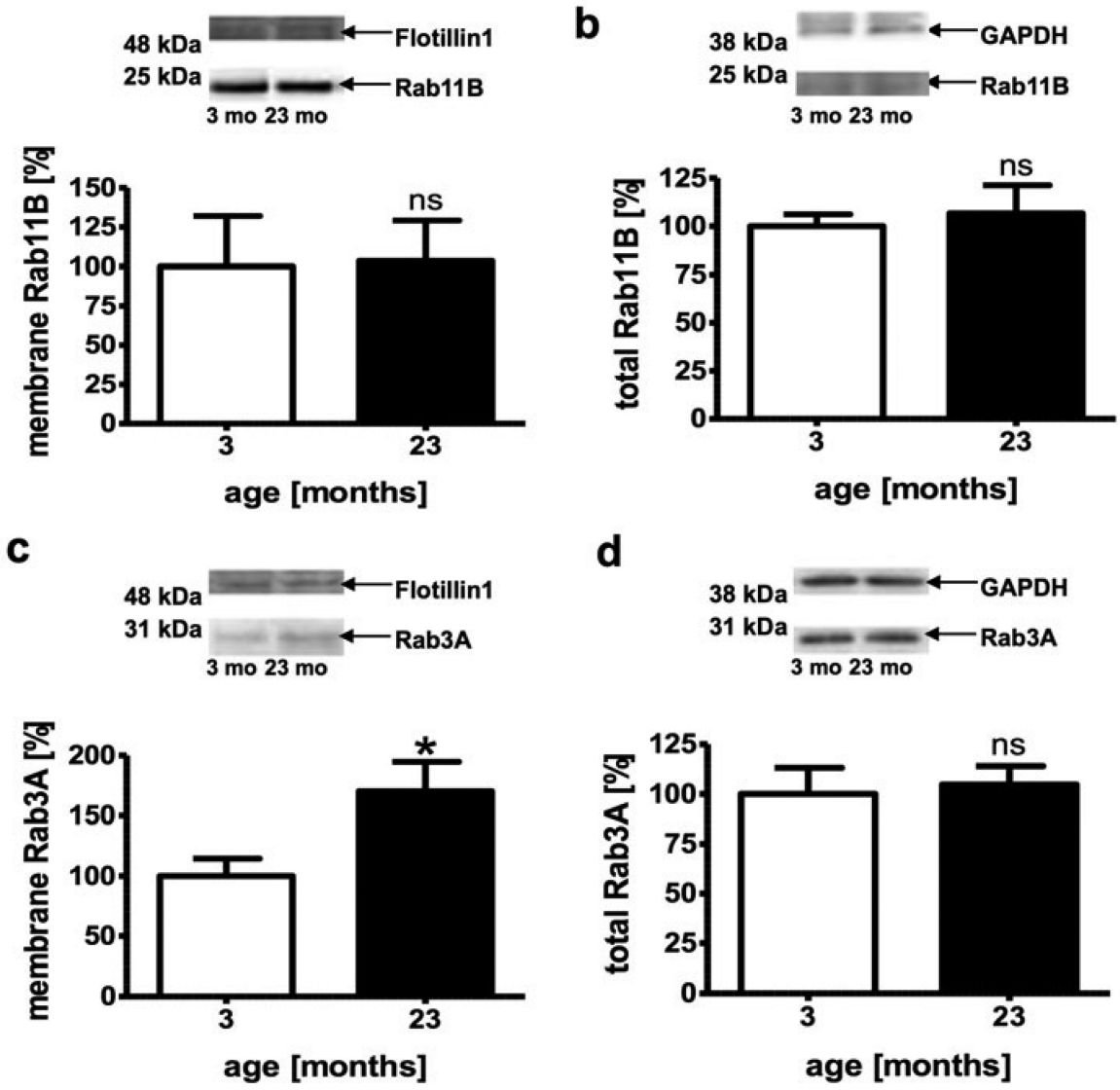


**Figure 2. Brain Rho-GTPase membrane-associated and total protein levels**

Western blot analysis of brain membrane preparations (a, c, e), and of total homogenates (b, d, f) isolated from brains of young (3 months old) and aged (23 months old) mice was used to characterize the prenylation of Rac1 (a, b), RhoA (c, d), and Cdc42 (e, f). Levels of membrane-associated, geranylgeranylated Rho-GTPases (membrane Rho-GTPases) were normalized to the membrane marker Flotillin1. Levels of total homogenates (total Rho-GTPases) were normalized to GAPDH. Each graph shows representive Western blots. Mean ±SEM, unpaired t-test (p*<0,05), n=6.

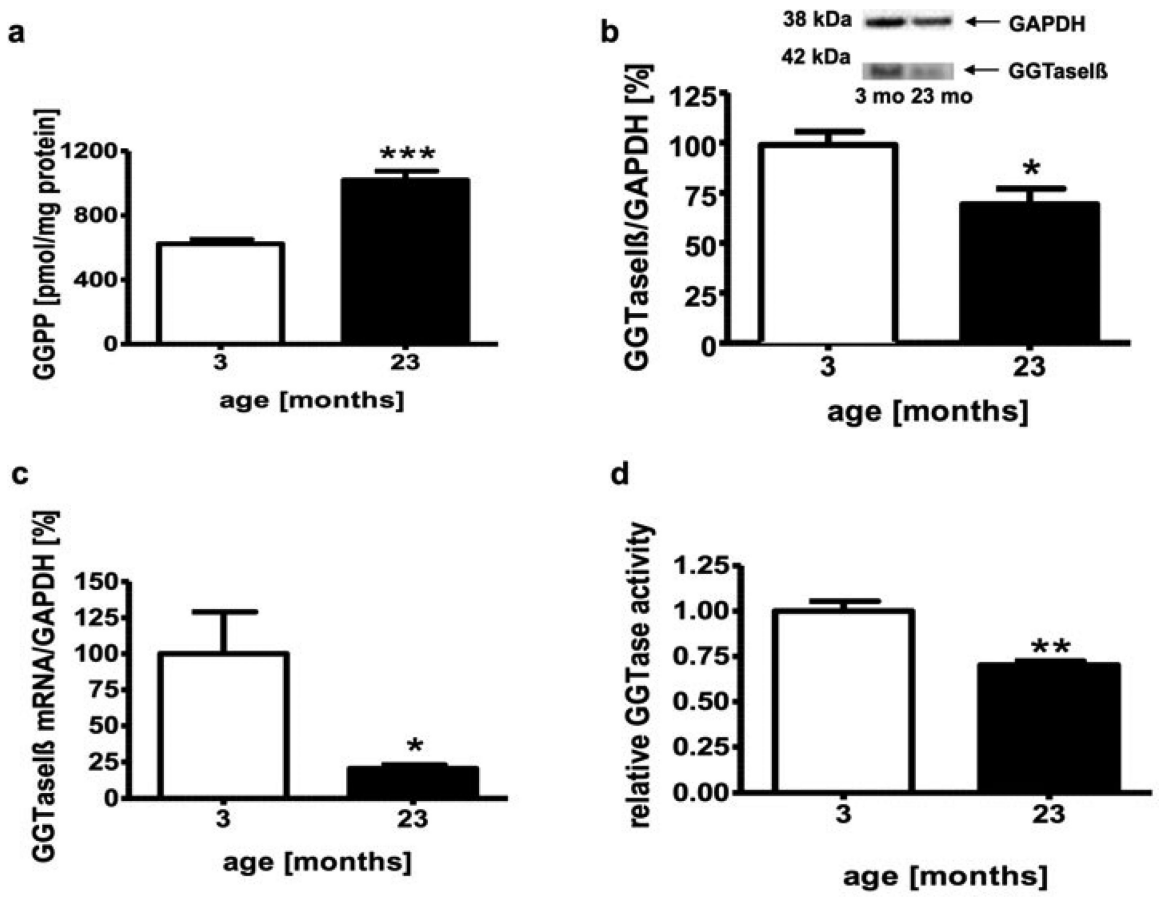


**Figure 3. Brain Rho/Rab-GTPase cytosolic protein levels**

Western blot analysis was used to characterize the the pool of of Rac1 (a), RhoA (b), Cdc42 (c), Rab11B (d) and Rab3A (e) in cytosolic fractions (isolated from brains of young (3 months old) and aged (23 months old) mice). Small GTPase levels were normalized to GAPDH. Each graph shows representive Western blots. Mean ±SEM, unpaired t-test (p*<0,05), n=6.

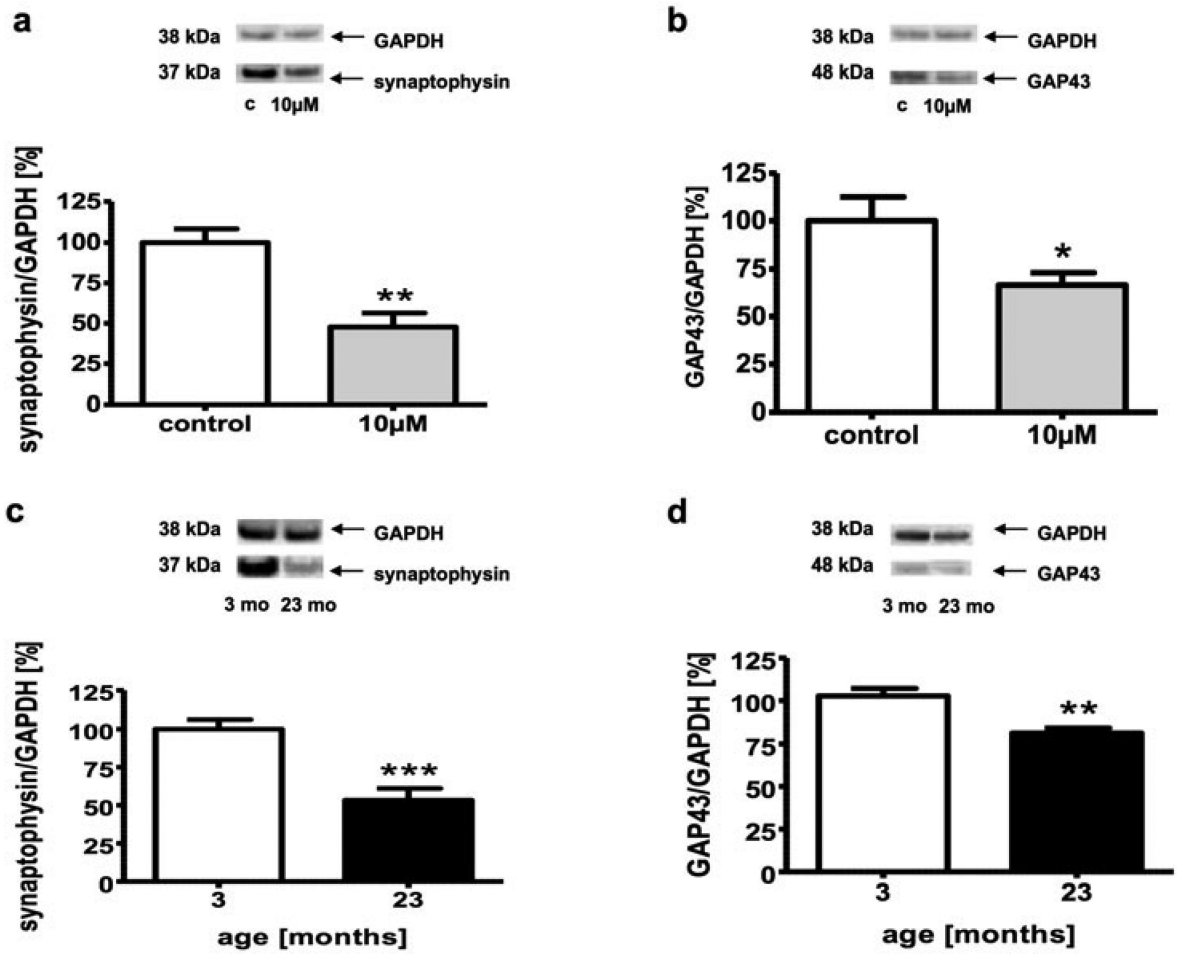


**Figure 4. Brain RhoGDIα and RabGDIα protein levels**

Western blot analysis of RhoGDIα (a) and RabGDIα (b) in brain cytosol preparations isolated from brains of 3 months old and 23 months old mice. Protein levels were normalized to GAPDH. Each graph shows representive Western blots. Mean ±SEM, unpaired t-test (ns=not significant), n=6.

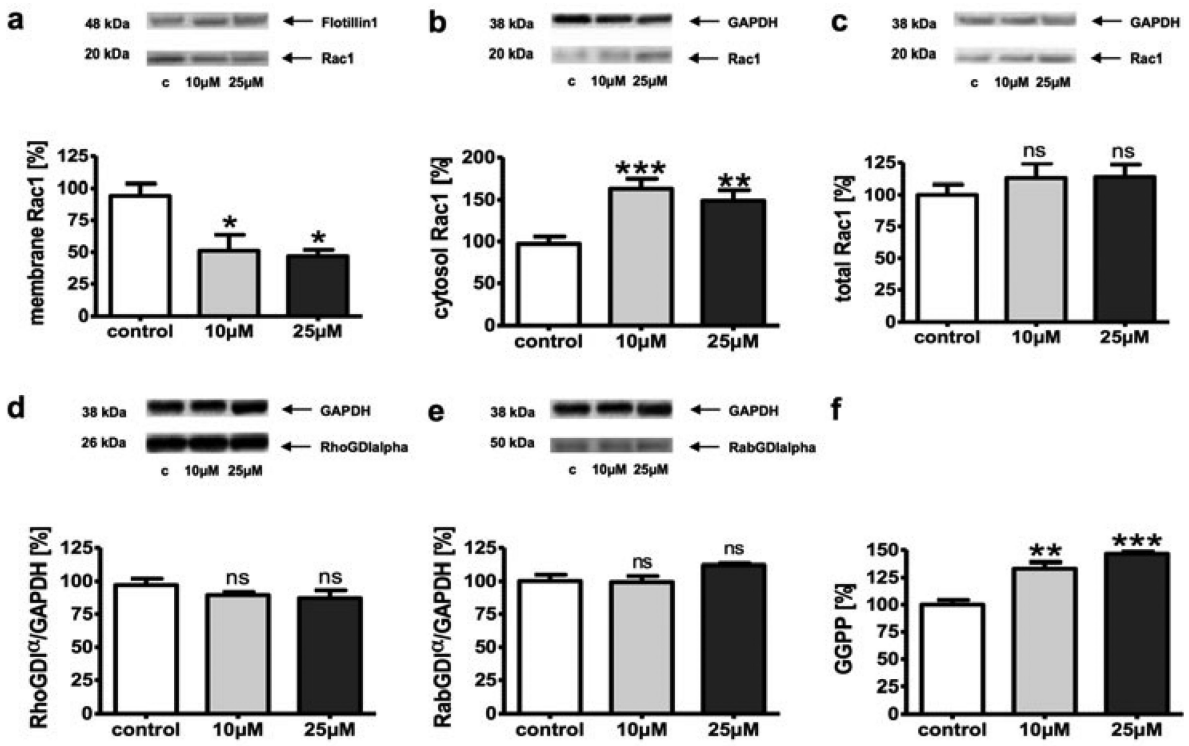


**Figure 5. Brain Rab-GTPase membrane-associated and total protein levels**

Western blot analysis of brain membrane preparations (a, c), and of total homogenates (b, d) isolated from brains of young (3 months old) and aged (23 months old) mice was used to characterize the prenylation of Rab11B (a, b) and Rab3A (c, d). Levels of membrane-associated, geranylgeranylated Rab-GTPases (membrane Rab-GTPases) were normalized to the membrane marker Flotillin1. Levels of total homogenates (total Rho-GTPases) were normalized to GAPDH. Each graph shows representive Western blots. Mean ±SEM, unpaired t-test (p*<0,05), n=6.

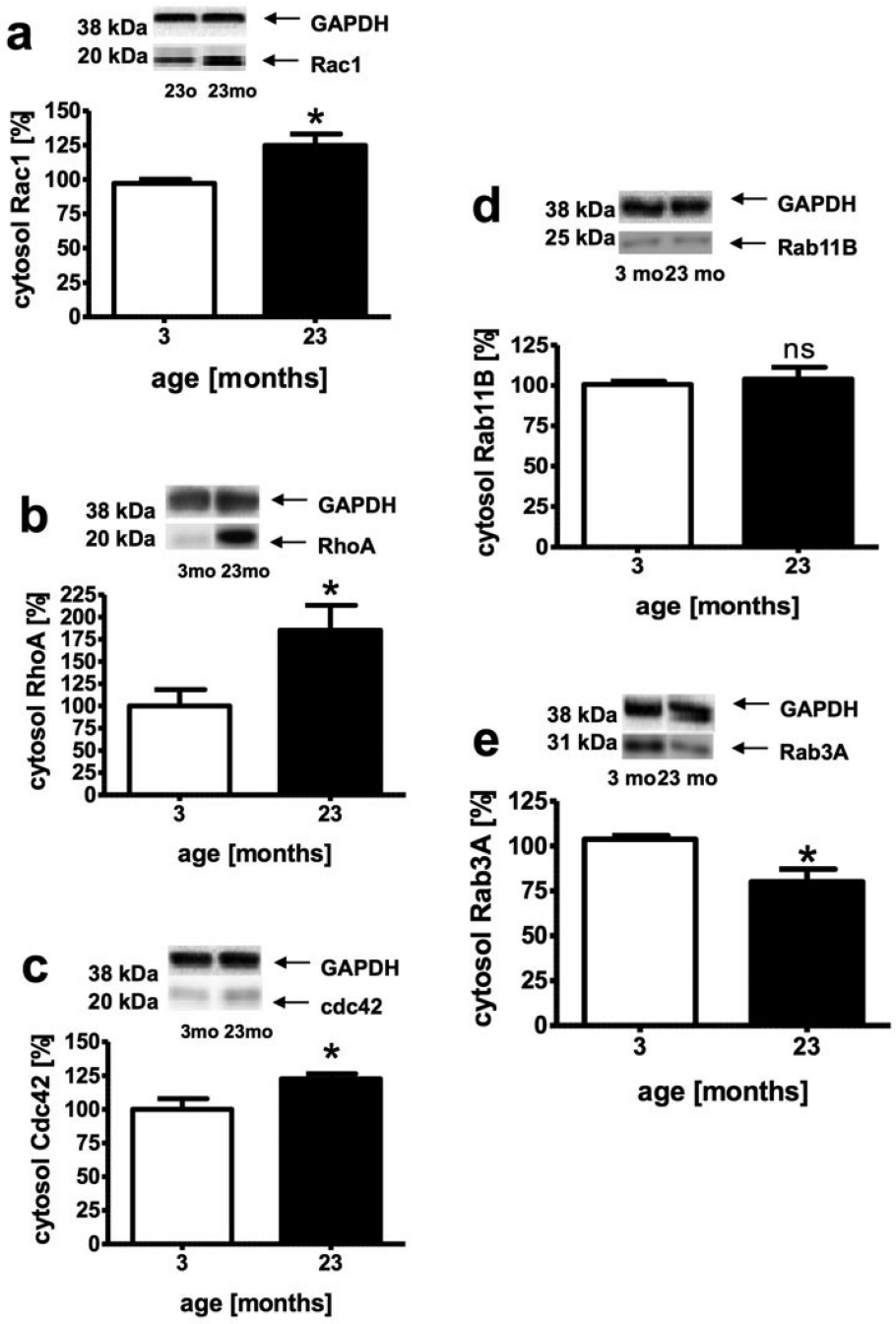


**Figure 6. Brain GGPP levels, relative GGTase activity, GGTase-I protein and gene expression**

(a) GGPP levels (pmol/mg protein) of brains from 3 and 23 months old C57BL/6mice were analysed using HPLC-FD as described in Material & Methods. Western blot analysis (b) and qRT-PCR (c) of brain homogenates isolated from brains of 3 and 23 months old C57BL/6 mice were used to assess protein (b) and mRNA (c) levels of the GGTase-I subunit. Relative GGTase activity (d) was assayed as described in the Materials & Methods section. GGTase-I protein and mRNA levels were normalized to GAPDH. Mean ±SEM, unpaired t-test ($p^{*}<0.05$; $p^{***}<0.001$), n=8 (GGPP levels), n=4 (relative GGTase activity), n=6 (GGTase-Iβ protein and gene expression levels).

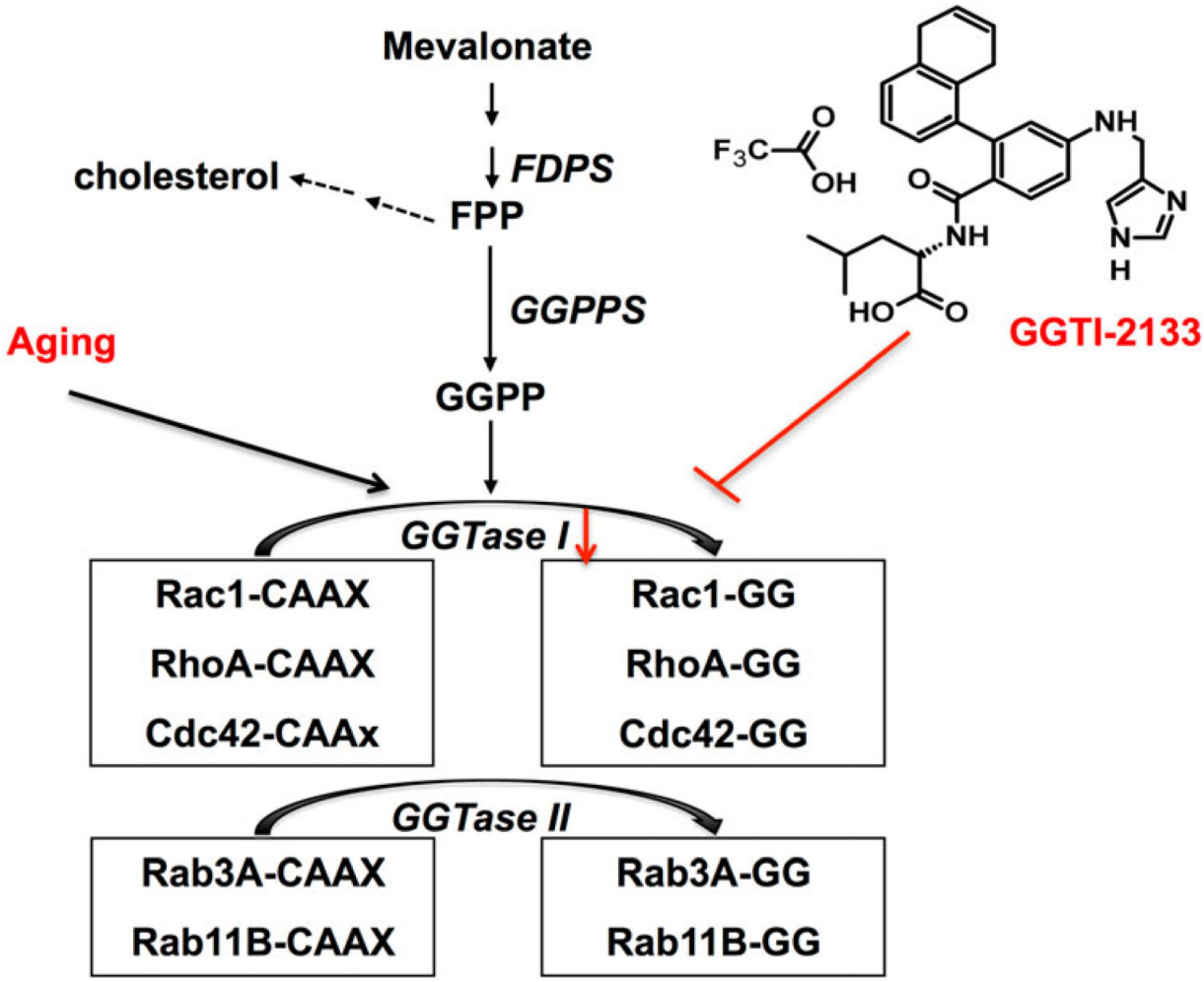


**Figure 7. Effects of GGTase-I inhibition on membrane localization of Rac1 and GGPP levels in human SH-SY5Y neuroblastoma cells**

Cells were treated with 10 and 25 μmol/L GGTI-2133 for 48 hrs. Western blot analysis of membrane preparations (a), cytosol preparations (b) and total homogenate (c) was used to characterize the cellular localization of Rac1 (a, b, c) in human SH-SY5Y neuroblastoma cells, treated with GGTI-2133. Levels of membrane-associated, geranylgeranylated Rac1 (membrane Rac1) were normalized to the membrane marker Flotillin1. Levels of cytosol and total Rac1 in cytosol preparations and cell homogenate were normalized to GAPDH. Western blot analysis of RhoGDIα(d) and RabGDIα(e) in cytosol preparations. Each graph shows representive Western blots. GGPP (f) levels were determined using HPLC-FD as described in Material & Methods. Mean ±SEM, p*<0.05; p**<0.01; p***<0.001; ns=not significant; ANOVA & Tukey's post test), n=6.

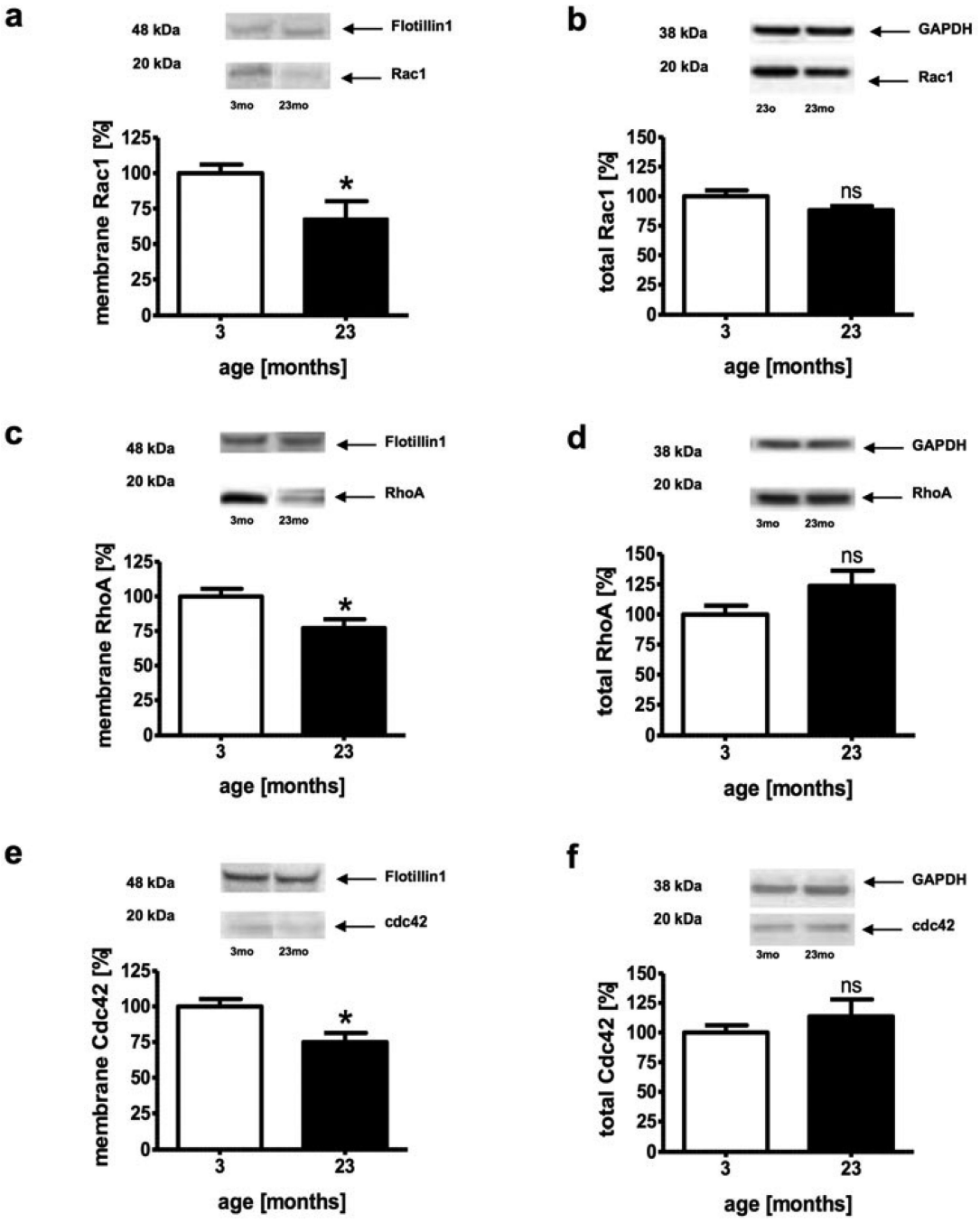


**Figure 8. Effects of GGTase-I inhibition on synaptic markers in SH-SY5Y neuroblastoma cells**

Cells were treated with 10 μmol/L GGTI-2133 for 48 hrs. Western blot analysis of total homogenates was used to assess protein levels of synaptophysin (a) and GAP43 (b). **Effects of aging on brain synaptic markers**. Western blot analysis of total homogenates isolated from brains of young (3 months old) and aged (23 months old) mice was used to assess protein levels of synaptophysin (c) and GAP43 (d). Protein levels were normalized to GAPDH. Each graph shows a representive Western blot. Mean ±SEM, unpaired t-test (p*<0.05; p**<0.01; p***<0.001), n=6.

**Cover image suggestion.** Rho-GTPases are geranylgeranylated by transferase GGTase-I. Their prenylation is essential for their localization in membranes, the site of their activation and function. Even that we found GGPP levels elevated in brains of aged

(23 months) mice compared to younger (3 months) mice as well as in GGTI-2133 treated SH-SY5Y cells, their amount of total (homogenate) Rho-GTPases (Rac1, RhoA and Cdc42) was unchanged. To our surprise the prenylated Rho-GTPases were decreased in membrane preparations of aged mice brains and SH-SY5Y, treated with the GGTaseI-inhibitor GGTI-2133. These findings directly correlate with the reduction of relative GGTase activity, GGTaseIβ protein and mRNA levels. Since Rac1, RhoA and Cdc42 are associated with synaptogenesis, we examined GAP43 and synaptophysin, two common synaptic marker proteins. GAP43 and synaptophysin declined in an age-related manner in the mouse brain and were also reduced in our in vitro model. Faulty regulation of Rho proteins in aged brain is associated with a specific deficit in GGTase-I , which could contribute to age-related deficits in neuronal outgrowth.