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INHIBITING GERANYLGERANYLATION INCREASES NEURITE BRANCHING AND DIFFERENTIALLY ACTIVATES COFILIN IN CELL BODIES AND GROWTH CONES

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Abstract

Inhibitors of the mevalonate pathway, including the highly prescribed statins, reduce the production of cholesterol and isoprenoids such as geranylgeranyl pyrophosphates. The Rho family of small guanine triphosphatases (GTPases) requires isoprenylation, specifically geranylgeranylation, for activation. Because Rho GTPases are primary regulators of actin filament rearrangements required for process extension, neurite arborization and synaptic plasticity, statins may affect cognition or recovery from nervous system injury. Here, we assessed how manipulating geranylgeranylation affects neurite initiation, elongation and branching in neuroblastoma growth cones. Treatment with the statin, lovastatin (20 μ M) decreased measures of neurite initiation by 17.0% to 19.0% when a source of cholesterol was present and increased neurite branching by 4.03 to 9.54 fold (regardless of exogenous cholesterol). Neurite elongation was increased by treatment with lovastatin only in cholesterol-free culture conditions. Treatment with lovastatin decreased growth cone actin filament content by up to 24.3%. In all cases, co-treatment with the prenylation precursor, geranylgeraniol (10 μ M), reversed the effect of lovastatin. In prior work, statin effects on outgrowth were linked to modulating the actin depolymerizing factor, cofilin. In our assays, treatment with lovastatin or geranylgeraniol decreased cofilin phosphorylation in whole cell lysates. However, lovastatin increased cofilin phosphorylation in cell bodies and decreased it in growth cones, indicating differential regulation in specific cell regions. Together, we interpret these data to suggest that protein geranylgeranylation likely regulates growth cone actin filament content and subsequent neurite outgrowth through mechanisms that also affect actin nucleation and polymerization.

Keywords

Actin microfilaments; Growth cones; Neurite outgrowth; Posttranslational modifications; Prenylation

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INTRODUCTION

Statins are pharmacological inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate limiting enzyme for the mevalonate pathway [1], that are widely prescribed to treat hypercholesterolemia and other lipid disorders [2-3]. Along with cholesterol, the mevalonate pathway produces protein modifying intermediates, including the 15 and 20 carbon isoprenoids, farnesyl and geranylgeranyl pyrophosphates (FPP, GGPP) [4]. Farnesyl transferase (FTase) and geranylgeranyl transferases (GGTase) I and II facilitate the transfer of farnesyl and geranylgeranyl moieties to cysteine residues of proteins having a C terminal CAAX domain, including the Ras superfamily of monomeric guanine triphosphatases (GTPases) [5-7]. Levels of GGPP increase in aged brain and Alzheimer's disease [8,9], and the action of GGTase I is required for plasticity [10]. Since members of the Rho subfamily of Ras GTPases are exclusively geranylgeranylated through the action of GGTase I [7], modulation of their isoprenylation may be important in preventing cognitive decline.

Synaptic remodeling requires arborization and extension of neuronal processes, followed by synapse formation and stabilization [11-13]. Rearrangement of the actin cytoskeleton in process tips (growth cones, developing synapses) is necessary for each of these mechanisms, and is primarily controlled by Rho subfamily GTPases (e.g. RhoA, Rac1, Cdc42) [10,14-19]. Rho GTPases regulate regional actin filament content through affecting actin nucleation, polymerization, severing and depolymerization [19]. Treatment with statins affects neurite outgrowth in several systems, but results vary according to the type of neuron and statin employed [10,20-27]. Interestingly, statin effects on neurite outgrowth are linked to manipulation of geranylgeranylation, rather than to decreases in cholesterol [10,20,21,24,27]. For example, atorvastatin decreases neurite initiation in PC12 cells and elongation in rat embryonic cortical neurons, effects rescued by geranylgeranylation precursors but not cholesterol [20,21]. However, pravastatin [22,23], simvastatin [22,28] and mevastatin [26] have all been reported to increase neurite outgrowth that is at least partly dependent on geranylgeranylation. In most of these studies, a single measure of outgrowth was reported, generally measuring neurite initiation as either the percent of neurite-bearing cells or the number of neurites/cell. In works addressing branching or elongation, statin treatment increases these measures [10,20,23]. The mechanisms leading to these results have yet to be fully defined, but several works suggest geranylgeranylation is important for regulating Rho GTPase and cofilin activity [27].

Here, we comprehensively assess the effects of inhibiting geranylgeranylation in a neuroblastoma cell line to distinguish isoprenoid from cholesterol effects and separate plasticity from degeneration. In the current work, we performed a comprehensive analysis of neurite outgrowth in the B35 neuroblastoma model system, evaluating neurite initiation, elongation and branching in response to manipulating geranylgeranylation, and assessing whether changes in growth cone actin filament content correlate with growth cone cofilin activity.

METHODS

Cell Culture and Treatment

B35 rat neuroblastoma cells (ATCC, Manassas, VA) were routinely maintained at 37°C in 1:1 Dulbecco's modified Eagle's medium and nutrient mixture F12 (DMEM/F12, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) and were passed when 90% confluent. For western blotting experiments, cells were seeded in 6 well or 10 cm tissue culture plates at 20,000 cells/cm²; for neurite outgrowth and immunochemical studies, cells were seeded on 12 or 25 mm glass coverslips coated overnight at 37°C with 25 µg/ml collagen (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) at a density of 5,000 cells/cm². To decrease production of isoprenoids, cells were treated for 24 hours with 20 µM lovastatin (gift from Merck, Whitehouse Station, NJ or from Sigma, St. Louis, MO). In lovastatin treated cells, co-treatment with 10 µM of the geranylgeranylation precursor, geranylgeraniol (GGOH, EMD Biosciences, San Diego, CA or Sigma, St. Louis, MO) was employed to promote protein geranylgeranylation [29]. Concentrations of lovastatin and geranylgeraniol were determined by evidence of morphological change within cultures in the absence of apparent toxicity (assessed using trypan blue exclusion and nuclear morphological changes) in dose response and time course analyses (data not shown). To provide a source of external cholesterol, experiments were generally performed with cells maintained in serum containing media (SCM). However, in some experiments cells were cultured in serum-free medium (SFM) or serum-free medium containing synthetic cholesterol (SFM+Chol, 1:500 dilution; Sigma, St. Louis, MO) to assess the role of cholesterol in neurite outgrowth.

Microscopy

Following treatments, cells were fixed in 4.0% paraformaldehyde for 20-30 minutes at room temperature. Fixed cultures were washed three times with PBS and either fluorescently labeled (see below) or directly mounted onto slides with mounting media containing 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Digital images (1 image per each replicate culture) were captured through either 40X or 100X objectives using a Zeiss Axiovert 200M interfaced with the Axiovision image analysis system (Zeiss, Jena, Germany) or a Nikon A1R-A1 confocal system with a Nikon Eclipse Ti inverted microscope and NIS elements imaging software (Nikon Instruments, Melville, NY). For fluorescently labeled cultures, image capture conditions, including lamp intensity and exposure time, were held constant.

Analysis of Neurite Outgrowth

Each image was assessed for the number of neurites/cell (for cells with neurites), the percent of neurite-bearing cells, the number of branch points/neurite and the total neurite length/cell. All quantifiable cells in each image were analyzed (8-15 cells/image). A cell was considered quantifiable if it and all its neurites were contained in the image. A neurite was defined as an extension greater than 10 µm; branches were bifurcations where each extension terminated in a growth cone; and the total neurite length was the sum of all neurites and branches from a single cell (quantified using only cells elaborating neurites). For all experiments, the sampling units were individual cultures. The number of sample replicates in each condition

varied from 1 to 4 per experiment. Data are reported as average values from each culture and were analyzed for differences across groups using two-way univariate analyses of variance (ANOVA) with treatment as a fixed factor and experiment replicate as a random factor at a significance level of $\alpha = 0.05$. When there was a significant main effect for treatment, differences from control cultures (SCM, SFM or SFM+Chol) were determined using the Least Significant Difference (LSD) or Tamhane's T2 post hoc test at $\alpha = 0.05$ (SPSS, Chicago, IL).

Immunocytochemistry and Actin Filament Labeling

Following fixation, cultures were blocked for 30 minutes in PBS containing 0.1% bovine serum albumin, 0.1% Triton X-100 and 1.5% pre-immune goat or donkey serum. For determining actin filament content, fixed cultures were incubated for 1 hour with 3.3 μ M alexafluor 488 conjugated phalloidin (Invitrogen, Carlsbad, CA), washed twice in PBS, and mounted onto slides. For immunolabeling of phospho-cofilin, fixed cultures were incubated overnight at 4°C with rabbit anti-phospho-S3 cofilin primary antibodies (1:150; AbCam, Cambridge, MA; catalog # ab12866). Following three 5 minute washes in blocking buffer, samples were incubated with donkey anti rabbit alexafluor 555 conjugated secondary antibodies (1:200, Invitrogen, Carlsbad, CA) for 30-60 minutes at room temperature. After two more 5-minute washes, cells were double-labeled using mouse anti-cofilin primary antibodies (1:100, AbCam, Cambridge, MA, catalog # ab54532) and goat anti-mouse alexafluor-488-conjugated secondary antibodies (5 μ g/ml, Invitrogen, Carlsbad, CA). The secondary antibody dilutions did not yield substantial non-specific binding in immunostaining controls processed without exposure to primary antibodies.

Immunocytochemical Image Analysis

From images captured through the 40X objective, regions of interest included cell bodies, neurites and growth cones. From images captured through a 100X objective, regions of interest included lamellipodia, filopodia, and central domains. From each image only isolated, intact regions of interest were quantified. For cultures labeled for filamentous actin, fluorescent intensities for regions of interest were averaged for each culture (sampling unit) and normalized to SCM control values for each experiment. Differences across treatment groups were compared using Kruskal-Wallis ANOVA across treatment. When there was a significant treatment effect, differences between individual treatments were determined using Mann-Whitney U post hoc tests. For double labeled images, the ratio of fluorescence intensity of phospho-cofilin to total cofilin staining was calculated for each region of interest and normalized to SCM control values in each experiment. Significant differences between treatment groups were determined using Kruskal-Wallis and Mann-Whitney U post hoc tests at a significance level of $\alpha = 0.05$ (SPSS, Chicago, IL).

Western Blotting

After treatment, Cells were lysed in 25 mM Tris-HCl (pH = 7.4), 150 mM NaCl, 1.5 mM EDTA, and 1.0% IGEPAL CA-630. Samples of cell lysates (20 μ g total protein) were electrophoresed (Mini protean III, BioRad, Temecula, CA) through 12% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels and then electrotransferred to nitrocellulose (0.2 μ m, BioRad, Temecula, CA). Membranes were blocked in 5% non-fat

milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for one hour and incubated overnight at 4°C with rabbit anti-phospho-S3 cofilin antibodies (1:200, Abcam, Cambridge, MA; catalog # ab12866) in TBST with 5% non-fat milk followed by extensive washing and exposure to horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (1:2500, Jackson ImmunoLabs, West Grove, PA) for 2 hours at room temperature. Blots were stripped and reprobed for total cofilin using either mouse or rabbit anti-cofilin antibodies (1:1000, Abcam, Cambridge, MA; catalog # ab18207, ab54532) and appropriate secondary antibodies conjugated to HRP (1:2500). Blots were visualized with SuperSignal west pico chemiluminescent substrate (Thermo Fisher, Waltham, MA). Efficient stripping was determined by exposing stripped blots to chemiluminescent substrate and exposure to film for 20 minutes. Immunoreactive bands were quantified using the integrated area for each band obtained from densitometric scans of film images (FluoroChem HD2, Alpha Innotech, San Leandro, CA). Ratios of densitometric values for anti-phospho-cofilin to anti-cofilin immunoreactive bands were calculated in each experiment ($n = 7$ in each condition) and assessed using ANOVA and Tamhane's post-hoc test at a significance level of $\alpha = 0.05$ (SPSS, Chicago, IL). In some experiments, we assessed equal protein loading by western blotting for actin. These controls consistently showed equal protein loading across treatment conditions (data not shown).

RESULTS

Inhibiting Geranylgeranylation Increases Neurite Branching

We first assessed how manipulating protein geranylgeranylation affected neurite initiation, elongation and arborization. To inhibit geranylgeranylation, cells were treated with the HMG CoA reductase inhibitor, lovastatin (LOV). To promote geranylgeranylation, cells in which the mevalonate pathway was inhibited with lovastatin were treated with the geranylgeranylation precursor, geranylgeraniol (GGOH), because GGOH can be converted to geranylgeranyl pyrophosphate and used for geranylgeranylation [30]. We assessed changes in outgrowth from cells cultured in serum containing medium (SCM, providing a source of cholesterol), serum-free medium (SFM, a condition known to induce outgrowth in B35 cells but not providing exogenous cholesterol), and SFM with the addition of a physiological concentration of synthetic cholesterol (SFM+Chol, Fig. 1). Compared to control cultures (Fig. 1a, d, g), cells treated with an optimal concentration of LOV (20 μ M; determined from maximal effect on cell morphology in absence detectable toxicity) induced cell body rounding and the elaboration of more highly branched neurites (Fig. 1b, e, h), effects that were generally reversed by co-treatment with 10 μ M GGOH (Fig. 1c, f, i).

Quantification of outgrowth indicated that protein geranylgeranylation limits neurite branching, and may promote neurite initiation. In analyzing the percent of neurite-bearing cells, there was a main effect for treatment ($F_{\text{TREAT}}(2, 21) = 10.197$, $p = 0.001$), experimental replicate ($F_{\text{EXP}}(7, 21) = 3.124$, $p = 0.033$) and their interaction ($F_{\text{TREAT*EXP}}(14, 21) = 4.295$, $p = 0.001$) in cells cultured in SCM, where treatment with LOV decreased the percent of neurite-bearing cells (Fig. 2a) by 19.0% (LSD posthoc, $p = 0.001$) compared to cells in SCM alone. When cells were co-treated with LOV and GGOH, the percent of neurite-bearing cells increased to 8.6% over SCM control values, and was

significantly different than cultures treated with LOV ($p = 0.001$). Similar results were found when analyzing the number of neurites/cell (Fig. 2b), which did not show significant main effects for treatment, experimental replicate or their interaction. However, because we had an a priori assumption that the LOV treatment group would be different than all other treatments, we ran pairwise comparisons. For cells cultured in SFM with added synthetic cholesterol, treatment with LOV non-significantly decreased the number of neurites/cell by 17.0%, compared to controls and co-treatment with LOV and GGOH increased the number of neurites/cell to 3.5% over control values ($p = 0.044$ compared to LOV). No differences were found in neurite initiation in among treatments in cells cultured in SFM without cholesterol (Fig. 2a, b).

Assessing neurite arborization using the number of branch points/neurite, there were significant main effects for the interaction between treatment and experiment under SCM ($F_{\text{TREAT}}(14, 21) = 9.696$, $p = 0.000$), for treatment ($F_{\text{TREAT}}(2, 12) = 68.787$, $p = 0.014$) and experiment ($F_{\text{EXP}}(1, 12) = 19.286$, $p = 0.048$) under SFM, and for treatment ($F_{\text{TREAT}}(2, 12) = 41.468$, $p = 0.024$) under SFM + Chol conditions (Fig. 2c). Here, treatment with LOV increased the number of branch points/neurite 9.54 times over SCM (LSD, $p < 0.001$), 4.30 over SFM (LSD, $p = 0.002$) and 4.03 fold over SFM+Chol (Tamhane's T_2 , $p = 0.054$), indicating that LOV increased neurite branching regardless of whether a source of exogenous cholesterol was present (Fig. 2c).

Neurite elongation, assessed as the total neurite length/cell in cells bearing neurites, did not have significant main effects of treatment, experiment or their interaction across any of the baseline conditions. However, the total neurite length/cell was increased 81.1% by treatment with LOV in cells cultured in SFM (Tamhane's T_2 , $p = 0.020$). A similar result was found when the length of the longest neurite was assessed. Here, treatment with LOV increased the length of the longest neurite/cell by 90.0%, compared to SFM controls (Tamhane's T_2 , $p = 0.008$).

Each of the outgrowth effects elicited by treatment with LOV was reversed when cells were co-treated with GGOH, indicating that the primary effects likely were realized through decreased protein geranylgeranylation. Interestingly, the presence of cholesterol was necessary to observe the effects of LOV on neurite initiation, but negatively impacted neurite elongation. Because the most dramatic result was the LOV induced increase in neurite branching, which was not affected by cholesterol, we elected to perform the next experiments in cells cultured in SCM.

Geranylgeranylation Promotes Growth Cone Actin Filament Accumulation

We next assessed how manipulating geranylgeranylation affects actin filament content in cells cultured in SCM. In subcellular regions (cell bodies, neurites, growth cones) defined from phase contrast images captured through a 40X objective, the average fluorescence intensity for each region under each treatment condition (SCM, LOV, LOV+GGOH) was determined from alexafluor 488 phalloidin labeled actin filaments (Fig. 3a-c). Non-parametric analysis of fluorescent intensities, normalized to SCM controls, indicated significant differences across treatments in neurites (Kruskal-Wallis $X^2_{(2, N=12)} = 6.217$, $p = 0.05$) and growth cones ($\chi^2_{(2, N=12)} = 5.899$, $p = 0.05$). Treatment with LOV (Fig. 3b)

decreased actin filament content in neurites and growth cones, by 24.3% (Mann-Whitney U, $Z = -2.460$, $p = 0.014$) and 21.6% (Mann-Whitney U, $Z = -2.46$, $p = 0.014$) respectively, compared to SCM control cultures (Fig. 3d). However, no changes in actin filament content were seen in cell bodies across treatments. In neurites and growth cones, co-treatment with LOV and GGOH returned the actin filament content to levels similar to those observed in control cultures maintained in SCM (Fig. 3d).

To further localize the site of decrease in growth cone actin filament content, we assessed phalloidin labeling in growth cone central domains, lamellipodia and filopodia, identified from differential interference contrast (DIC) images captured through a 100X objective. Quantification of average fluorescent intensities from phalloidin staining of cells in each condition (SCM, LOV, LOV+GGOH; Fig. 4a-c) indicated significant differences across treatment conditions in growth cone central domains (Kruskal-Wallis, $X^2_{(2, N=9)} = 7.448$, $p = 0.024$) but not lamellipodia or filopodia. In central domains, treatment with LOV reduced actin filament content by 16.2%, compared to control (Mann-Whitney U, $Z = -2.087$, $p = 0.037$) and co-treatment with GGOH not only reversed the effect of LOV, but further increased actin filament content levels above SCM for central domains (Mann-Whitney U, $Z = -2.087$, $p = 0.037$) and lamellipodia (Mann-Whitney U, $Z = -2.087$, $p = 0.037$), but not for filopodia (Fig. 4d). Thus, it appears that decreasing geranylgeranylation correlates with a decrease in actin filament content specifically in cellular areas associated with process outgrowth and branching (e.g. growth cone central domains and lamellipodia).

Manipulating Geranylgeranylation Differentially Affects Cofilin Activity in Subcellular Regions

Changes in actin filament content could occur through altering actin polymerization or depolymerization rates. Because it was earlier demonstrated that activation of the actin depolymerizing factor, cofilin, is affected by manipulating the mevalonate pathway, we next determined whether this holds true in our system. Activation of cofilin is inhibited by phosphorylation at the serine residue at amino acid position 3 (S3) [31]. Immunoblots from whole cell lysates, indicated that treatment with LOV for 2 or 24 hours or exposure to LOV +GGOH for 24 hours altered phospho-cofilin levels (representative blot, Fig. 5a), while total levels of cofilin remained relatively unchanged over this time frame (blot from same representative experiment, Fig. 5b). After 2 hours of treatment, quantification by scanning densitometry indicated that treatment with LOV decreased the ratio of phospho-cofilin to anti-total cofilin by 24.6%, an increase in cofilin activation that reached 36.5% by 24 hours of treatment (Fig. 5c). In contrast, co-treatment with LOV and GGOH had little effect on cofilin phosphorylation at 2 hours (Fig. 5c); however, 24 hour exposure to LOV+GGOH decreased the ratio of phospho-cofilin to total cofilin by 36.4% (Fig. 5c).

These data are consistent with those measuring actin filament content in neurites and growth cones (particularly central domains) in that treatment with LOV, which activates cofilin, correlates with a decrease in actin filament content. It is possible that cofilin activity may be substantially affected in subcellular regions. To address this, we also assessed the ratio of phospho-cofilin to total cofilin by immunocytochemistry. From images captured through a 40X objective, we identified regions of interest (cell bodies, neurites, growth cones) from

phase contrast images and determined the ratio of phospho-cofilin to cofilin immunoreactivity using average fluorescence intensities. We expected the ratio of phospho-cofilin to cofilin to decrease in cell bodies, reflecting the assumption that cell bodies constituted the bulk of the whole cell lysates. There were significant differences across treatments in cell bodies (Kruskal-Wallis, $X^2_{(2, N=12)} = 7.565$, $p = 0.023$) and growth cones ($X^2_{(2, N=12)} = 7.449$, $p = 0.024$). However, contrary to our expectation, the ratio of phospho-cofilin to cofilin increased by 50.5% (Mann-Whitney U ($Z = -2.323$, $p = 0.02$) in cell bodies and decreased by 31.9% in growth cones ($Z = -2.323$, $p = 0.02$, Fig. 6). We interpret this result to indicate that manipulating protein geranylgeranylation reflects either: (1) the major contribution of cofilin activity occurs in growth cones when measured through western blotting (see Fig. 5; e.g. the majority of cofilin is located in the growth cone region); or (2) decreases in growth cone actin filament content (see Fig. 3, 4) result from the activity of actin nucleating and polymerizing factors as well as the action of cofilin. To address the first concern, we assessed the phospho-cofilin to cofilin ratio, specifically in growth cone subregions (central domains, lamellipodia and filopodia). We did not find any significant differences between treatment conditions in this assessment (Fig. 7). Thus, it seems likely that actin nucleators and/or polymerization factors are involved in the modulation of actin dynamics and growth cone migration during outgrowth and arborization.

DISCUSSION

We performed the present study to further define the mechanisms through which protein geranylgeranylation regulates process outgrowth and arborization by directing actin cytoskeletal rearrangements in growth cones. The work was also designed to address previously reported results that were inconclusive on statin influences on outgrowth [10,20-27] and potential mechanisms through which statins mediate their effects. We found that inhibiting geranylgeranylation: (1) decreases neurite initiation under conditions where cholesterol is present; (2) increases neurite branching in the presence or absence of an exogenous source of cholesterol; and (3) decreased actin filament content in neurites and growth cones. Since decreases in actin filament content could result from increased activation of the actin depolymerizing factor, cofilin, we also assessed cofilin activity in whole cells and subcellular regions. Interestingly, we found that cofilin activation did not correlate with growth actin filament content, which decreased with inhibition of geranylgeranylation (by the HMG CoA reductase inhibitor, LOV) and increased in growth cone central domains and lamellipodia when geranylgeranylation was promoted (with co-treatment of LOV and GGOH).

The neurite outgrowth results reported here agree with prior studies showing that statin effects on neurite outgrowth are primarily attributable to decreased protein isoprenylation [10,20,21,24,27]. However, some studies indicate that, similar to our results, statins decrease neurite initiation [20], but others report that statins increase neurite initiation [23,26,27]. The basis behind these differences is unknown, but may reflect differences in models, applied statins or assay conditions. We were unable to determine any pattern across different model systems. For example, studies employing PC12 cells under similar growth conditions variably showed that statin treatment could increase [20,27] or decrease outgrowth [20]. Interestingly, prior studies conducted with the statin, atorvastatin, indicated a decrease in

neurite outgrowth [20,21], while those employing pravastatin [22,23], simvastatin [22,28] or mevastatin [26] all reported either enhanced or unaffected outgrowth. This may indicate a difference between the actions of fermentation-derived (type 1; pravastatin, simvastatin, mevastatin) and synthetic (type 2; atorvastatin) statins on specific isoprenylated targets. However, our results with lovastatin, a fermentation-derived statin, stand in contradiction to those reported by Fernandez-Hernando and colleagues (2005), a group who did use a different model system [27]. Thus, it is likely that the pleiotrophic effects of statins result in a complex regulation of protein isoprenylation and cholesterol production to influence neuronal process extension.

We interpret our data as indicating that modulation of protein geranylgeranylation is the primary mode through which inhibiting the mevalonate pathway affects growth cone actin filament content and outgrowth. Proteins with a primary sequence having a C terminal CAAX motif can be isoprenylated, with farnesylation occurring when X is serine or threonine and geranylgeranylation occurring when X is leucine or phenylalanine [5]. Because our outgrowth and actin filament content results depend on alterations in geranylgeranylation, we focused on signaling from Rho family GTPases, important regulators of actin dynamics and outgrowth that are exclusively geranylgeranylated by GGTase I. Activation (GTP loading) of the Rho GTPases occurs through interaction with GEFs, many of which associate with the plasma membrane [7]. Thus, translocation of Rho GTPases to the plasma membrane is thought to be required for their activation [32,33]. If this is true, then regulation of Rho GTPase membrane localization may play an important role in compartmentalizing activation of specific Rho GTPases. This might explain differential activation of RhoA and Rac1 in growth cones compared to whole cell lysates [34]. Additionally, regulation of Rho GTPase membrane localization may be important in determining the role of GTPase crosstalk and specific roles during outgrowth. This will help determine the source of differences seen with traditional dominant active and dominant negative Rho GTPase constructs, where activated Rac1 and Cdc42 generally promote process extension and RhoA promotes retraction [14], and more recent studies indicating that all three proteins play prominent roles in directing efficient extension of neuronal processes [15-17].

Previous work implicated the actin depolymerizing factor, cofilin, as mediating outgrowth alterations induced by statins [27]. In whole cells, we found that both inhibiting and promoting geranylation decreased cofilin phosphorylation, which indicates increased cofilin activity. Cofilin activity is critical for growth cone motility and neurite extension [35-37] and leads to decreased filamentous actin in cell bodies and generates free barbed ends *in vivo* [38]. Rho GTPases decrease cofilin activity by activating LIN-11, Islet-1, MEC-3 domain (LIM) kinases [39]. LIM kinases are activated by phosphorylation by either Rho coiled-coiled (ROCK, via RhoA) or p21-activated (PAK, via Rac1 and Cdc42) kinases [40]. However, the correlation between decreased actin filament content and activation of cofilin did not hold in our immunocytochemical study, where growth cone cofilin phosphorylation decreased (indicating increased cofilin activity) with treatment to inhibit (LOV) or promote (LOV+GGOH) protein geranylgeranylation, whereas LOV decreased growth cone actin filament content, but co-treatment with LOV and GGOH increased growth cone actin

filament content. This suggests that mechanisms promoting actin nucleation and polymerization are additionally activated during co-treatment with LOV and GGOH [19]. Rho GTPases promote actin nucleation and polymerization activation by activating formins (e.g. mDia1, activated by Cdc42 and RhoA) or members Wiskott-Aldrich Syndrome Protein (WASP) family (e.g. n-WASP and WAVE, activated by Cdc42 and Rac1, respectively) [41-44]. This likely contributes to the mechanism through which we observe the most dramatic changes in neurite outgrowth, process branching. Neurite branching is reported to require increased actin polymerization [45], and may also be associated with Rho GTPase regulated activation of cofilin, which also increases neurite branching [35], likely through producing more actin filament ends to facilitate polymerization [38].

Finally, treatment with statins has been reported in some studies to decrease the incidence of Alzheimer's disease or cognitive decline in aging [46]. While some of the effects are related to decreased cholesterol [47], there is good evidence that the decrease in protein isoprenylation may also play an important role [46,48,49]. Our data suggest that statins increase neurite arborization, an aspect of neurite outgrowth that likely correlates with increased synaptic plasticity. If this is true, we would expect statins to protect cognitive decline by decreasing the amount of farnesylated or geranylgeranylated proteins. This hypothesis is consistent with studies showing that statins enhance long-term potentiation [48], and demonstrating that decreasing prenyltransferases like farnesyl transferase ameliorate cognitive decline in a mouse model of Alzheimer's disease [49]. It will be interesting to learn whether similar results are seen with geranylgeranyl transferase inhibitors.

The results reported here support a hypothesis where inhibiting geranylation promotes neurite arborization, in a process associated with decreased growth cone actin filament content and activation of cofilin. However, regulation of actin polymerization appears to be important for outgrowth under conditions where protein geranylgeranylation is promoted. It is interesting to speculate that reports of statins decreasing the incidence of Alzheimer's disease or cognitive decline in aging might be related to statin-induced process arborization. Thus, additional studies are required to address the implications of statin-induced arborization in establishing neuronal networks and the effects on synaptic plasticity. In all, the results reported here suggest that mechanisms regulating Rho GTPase signaling, and subsequent effects on growth cone actin dynamics, need to be further elucidated to better define how neurons develop and establish synaptic connections, as well as determine novel sites for targeted intervention for degenerative or traumatic nervous system lesions.

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Fig. 1.

Geranylgeranylation alters B35 cell morphology. Representative differential interference contrast (DIC) images captured through a 40X objective showing cellular morphology of B35 cells cultured under control conditions including maintenance in serum containing medium (SCM) alone (a), in serum free medium (SFM) with no added cholesterol (d), or in SFM plus synthetic cholesterol (g). Compared to these baseline conditions, cells treated with 20 μ M lovastatin (LOV; b, d, h) for 24 hours led to cells with rounder cell bodies, fewer neurites and prominent neurite branching. Co-treatment with 20 μ M LOV and 10 μ M geranylgeraniol (Lov+GGOH; c, f, i) return cells to morphology similar to that seen in control conditions. Scale bar in a = 20 μ m and is valid for all images

Fig. 2.

Geranylgeranylation limits neurite branching. B35 cells were cultured under control conditions (black bars in all graphs), including maintenance in serum containing medium (SCM, $n = 17$ individual cultures from 8 separate experiments), serum-free medium without added cholesterol (SFM, $n = 5$ individual cultures from 2 separate experiments) and SFM with synthetic cholesterol added (SFM+Chol, $n = 5$ individual cultures from 2 separate experiments) and either not treated further or were treated for 24 hours with 20 μ M lovastatin (LOV, gray bars; $n = 16$, 6 and 6 individual cultures from 8 separate experiments for SCM, SFM and SFM+Chol, respectively) or 20 μ M LOV plus 10 μ M geranylgeraniol (LOV+GGOH, white bars; $n = 12$, 6 and 6 individual cultures from 8 separate experiments for SCM, SFM and SFM+Chol, respectively). Cultures were fixed and evaluated by image analysis for measures of different aspects of neurite outgrowth, including initiation using the percent of neurite-bearing cells (a) and the number of neurites/cell (b), arborization using the number of branch points/neurite (c), and elongation using the total neurite length/cell (d) for cells with neurites. Data are means $\pm$ SEM. In each culture, 6-17 cells were analyzed for a total of 36-62 analyzed in each treatment condition. * indicates significant difference compared to control cultures (SCM, SFM or SFM+Chol) or LOV+GGOH at $p < 0.05$ (ANOVA with LSD or Tamhane's T2 post-hoc)

Fig. 3.

Geranylgeranylation supports accumulation of actin filaments in neurite and growth cones. From regions of interest identified from representative phase contrast images (a), average fluorescence intensities for alexafluor 488 conjugated phalloidin labeled actin filaments were determined from corresponding images captured through a fluorescein isothiocyanate (FITC) filter set (b). Compared to cells maintained in serum containing medium (SCM, $n = 4$ individual cultures with a total of 70 cell bodies, 77 neurites and 73 growth cones), treatment with 20 μM lovastatin (LOV, $n = 4$ individual cultures with 75 cell bodies, 76 neurites and 68 growth cones) for 24 hours decreased the actin filament content in growth cones and neurites, but not cell bodies (c). Co-treatment with GGOH ($n = 4$ individual cultures with a total of 68 cell bodies, 71 neurites and 70 growth cones). Scale bar in a = 20 μm and is valid for images in a and b. Data in c are medians $\pm$ interquartile ranges for 4 replicate cultures in each condition, normalized to the overall mean intensity for SCM control in each region of interest. * indicates significant difference from controls (SCM condition in each experiment) and LOV+GGOH at $p = 0.05$ (Kruskal-Wallis with Mann-Whitney U post hoc)

Fig. 4.

Geranylgeranylation supports accumulation of actin filaments in growth cone central domains and lamellipodia. From regions of interest identified from representative differential interference contrast (DIC) images (a), average fluorescence intensities for alexafluor 488 conjugated phalloidin labeled actin filaments were determined from corresponding images captured through a fluorescein isothiocyanate (FITC) filter set (b). Compared to cells maintained in serum containing medium (SCM, $n = 3$ individual cultures with a total of 15 central domains, 15 lamellipodia and 28 filopodia), treatment with LOV did not alter actin filament content in growth cone subregions ($n = 3$ individual cultures with a total of 15 central domains, 15 lamellipodia and 15 filopodia). Co-treatment with 20 μ M lovastatin and 10 μ M geranylgeraniol (LOV+GGOH) for 24 hours increased the actin filament content in growth cone central regions ($n = 3$ individual cultures with a total of 15 central domains) and lamellipodia ($n = 3$ individual cultures with a total of 14 lamellipodia), but not filopodia ($n = 3$ individual cultures with a total of 22 filopodia; c). Scale bar in a = 10 μ m and is valid for images in a and b. Data in c are medians $\pm$ interquartile ranges for 3 replicate cultures in each condition, normalized to the overall mean intensity for SCM control in each region of interest. * indicates significant difference from controls (SCM) and # indicates significant difference from lovastatin-treated (LOV) at $p = 0.05$ (Kruskal-Wallis and Mann-Whitney U post-hoc)

Fig. 5.

Manipulating geranylgeranylation decreases cofilin phosphorylation in whole cell lysates. B35 cells were either maintained in serum containing medium (SCM) or treated with 20 μ M lovastatin (LOV) or 20 μ M LOV plus 10 μ M geranylgeraniol (LOV+GGOH) for 2 or 24 hours, followed by western blotting for phospho-cofilin (a, representative blot) and total cofilin (b, blot from same experiment as representative blot in a). Western blot were quantified using scanning densitometry and graph in c depicts means $\pm$ SEM of ratios of the densitometric values obtained from anti-phospho-cofilin reactive bands to anti-cofilin immunoreactive bands in each experiment, normalized to the mean of SCM controls. * indicates significant difference from control at $p < 0.05$ (ANOVA with Tamhane's post hoc; $n = 5$ separate experiments with 1 culture/treatment condition in each experiment)

Fig. 6.

Inhibiting or promoting geranylgeranylation increases phospho-cofilin in cell bodies and decreases phospho-cofilin in growth cones. In cells cultured in serum-containing medium (SCM, $n = 3$ individual cultures from 3 separate experiments), treatment with 20 μM lovastatin without (LOV, $n = 3$ individual cultures from 3 separate experiments) or with 10 μM geranylgeraniol (LOV+GGOH, $n = 3$ separate cultures from 3 separate experiments), the ratio of phospho-cofilin to total cofilin increased in cell bodies (total number analyzed = 46 for SCM, 49 for LOV and 26 for LOV+GGOH) and decreased in growth cones (total number analyzed = 43 for SCM, 39 for LOV and 41 for LOV+GGOH), without altering cofilin activation in neurites (total number analyzed = 48 for SCM, 45 for LOV and 47 for LOV+GGOH). Data are medians $\pm$ interquartile ranges ($n = 3$ separate experiments), * indicates significant difference from untreated cells at $p < 0.05$ (SCM; Kruskal-Wallis with Mann-Whitney U post hoc)

Fig. 7.

Manipulating geranylgeranylation does not alter phospho-cofilin levels in growth cone central domains, lamellipodia or filopodia. In cells cultured in serum-containing medium (SCM, $n = 3$ individual cultures from 3 separate experiments), treatment with 20 μM lovastatin (LOV, $n = 3$ individual cultures from 3 separate experiments) or LOV plus 10 μM geranylgeraniol (LOV+GGOH, $n = 3$ individual cultures from 3 separate experiments) not show any significant changes in the ratio of phospho- to total cofilin in growth cone central domains (total analyzed = 15 for each treatment), lamellipodia (total number analyzed = 15 for each treatment) or filopodia (total number analyzed = 24 for SCM, 28 for LOV and 27 for LOV+GGOH), compared to cells maintained in SCM. Data are medians $\pm$ interquartile ranges ($n = 3$ separate experiments)