Protein Prenylation Constitutes an Endogenous Brake on Axonal Growth

Graphical Abstract

Highlights

- Statins are the most potent enhancers of neurite elongation in vitro
- Statins and protein prenylation inhibitors promote CNS axon regeneration in vivo
- Motor neurons of early-onset ALS patients express high levels of prenylation enzyme
- Prenylation inhibitors provide a potential therapeutic approach for CNS regeneration

Authors

Hai Li, Takaaki Kuwajima, Derek Oakley, ..., Marie T. Filbin, Brent Stockwell, Christopher E. Henderson

Correspondence

chris.henderson@biogen.com

In Brief

Using a high-throughput phenotypic screen, Li et al. identify statins and inhibitors of protein prenylation as potent neurite-outgrowth-promoting agents. High levels of prenylation enzyme are found in patients with earlier-onset forms of ALS. Prenylation may limit axonal growth in both normal and pathological situations.
Protein Prenylation Constitutes an Endogenous Brake on Axonal Growth

Hai Li,1,3 Takaaki Kuwajima,1,3 Derek Oakley,5,11 Elena Nikulina,4 Jianwei Hou,4 Wan Seok Yang,1,5,12 Emily Rhodes Lowry,8 Nuno Jorge Lamas,3,6,7 Mackenzie Weygandt Amoroso,5,13 Gist F. Croft,4,14 Raghavendra Hosur,9 Hynek Wichterle,1,3,6 Said Sebti,8 Marie T. Filbin,4 Brent Stockwell,1,5 and Christopher E. Henderson1,2,3,6,10,15,*

1Center for Motor Neuron Biology and Disease, Columbia Stem Cell Initiative, Columbia Translational Neuroscience Initiative, Columbia University, New York, NY 10032, USA
2Department of Rehabilitation and Regenerative Medicine
3Department of Pathology and Cell Biology, Neurology, and Neuroscience
College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA
4Department of Biological Sciences, Hunter College, City University of New York, NY 10065, USA
5Howard Hughes Medical Institute and Department of Biological Sciences and Department of Chemistry, Columbia University, New York, NY 10027, USA
6Project A.L.S./Jenifer Estess Laboratory for Stem Cell Research, New York, NY 10032, USA
7Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Minho, Portugal
8Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, FL 33612, USA
9Computational Biology, Biogen Inc., Cambridge, MA 02142, USA
10Target ALS Foundation, New York, NY 10032, USA
11Present address: Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, USA
12Present address: Department of Biological Science, St. John’s University, Jamaica, NY 11439, USA
13Present address: Center for Brain Science, Harvard University, Cambridge, MA 02138, USA
14Present address: Laboratory of Stem Cell Biology and Molecular Embryology, Rockefeller University, New York, NY 100665, USA
15Present address: Neurology Research, Biogen, Inc., Cambridge, MA 02142, USA
*Correspondence: chris.henderson@biogen.com
http://dx.doi.org/10.1016/j.celrep.2016.06.013

SUMMARY

Suboptimal axonal regeneration contributes to the consequences of nervous system trauma and neurodegenerative disease, but the intrinsic mechanisms that regulate axon growth remain unclear. We screened 50,400 small molecules for their ability to promote axon outgrowth on inhibitory substrata. The most potent hits were the statins, which stimulated growth of all mouse- and human-patient-derived neurons tested, both in vitro and in vivo, as did combined inhibition of the protein prenylation enzymes farnesyltransferase (PFT) and geranylgeranyl transferase I (PGGT-1). Compensatory sprouting of motor axons may delay clinical onset of amyotrophic lateral sclerosis (ALS). Accordingly, elevated levels of PGGT1B, which would be predicted to reduce sprouting, were found in motor neurons of early- versus late-onset ALS patients postmortem. The mevalonate-prenylation pathway therefore constitutes an endogenous brake on axonal growth, and its inhibition provides a potential therapeutic approach to accelerate neuronal regeneration in humans.

INTRODUCTION

Axonal growth is an essential step in the formation of neural circuits during normal development. In cases of traumatic brain injury or neurodegenerative disease, axonal damage is among the first morphological manifestations, and suboptimal regeneration is thought to be a major contributor to the low rates of functional recovery. Enhancing axonal regeneration in a controlled manner in patients with spinal cord injury may allow them to regain key lost functions; similar treatment in patients with amyotrophic lateral sclerosis (ALS) has the potential to increase muscle strength over the course of the disease. Over the past few decades, we have gained considerable insight into the extrinsic factors and intrinsic signaling mechanisms that affect directional choices taken by the axon growth cone and govern its reduced ability to advance in the damaged CNS (Alizadeh et al., 2015). However, we still have little knowledge of the cell-intrinsic mechanisms that drive axonal forward growth.

Over the past few decades, much insight has been gained into the low regenerative capacity of the adult CNS (Liu et al., 2011; Yiu and He, 2006). Most studies have focused on extrinsic factors that restrict axon regeneration (Filbin, 2003; Thiede-Stan and Schwab, 2015). Three growth inhibitors—myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte myelin glycoprotein—act through a common receptor complex containing the ligand-binding Nogo-66 receptor (NgR) and its
Figure 1. High-Throughput Screen for Small Molecules that Enhance Neurite Outgrowth on Inhibitory Substrata Identifies Statins as the Most Potent Hits

(A) Fields of live mouse ES-MNs expressing the Hb9::GFP transgene imaged through monolayers of control CHO (Ctrl-CHO) or MAG-CHO cells. False colors are assigned to individual neurons by MetaMorph, which calculates neurite outgrowth parameters on a cell-by-cell basis. Neurite outgrowth on MAG-CHO cells is severely restricted, but cell survival is little affected. Scale bar, 100 μm.

(B) ES-MNs and E12.5 primary mouse motor neurons show comparable growth inhibition on MAG-CHO (mean ± SEM, n = 8; p < 0.005 for both ES-MNs and primary MNs).

(C) Schematic of the high-throughput screen.

(D) A snapshot of a fraction of the screening data. Neurite outgrowth data were converted to fold-increase by normalizing them to the negative controls in each plate. Data collected from forty 96-well plates are plotted for negative and positive controls, as well as test compounds.

(E) Compound libraries used for the screen. The number of compounds screened, primary hit rate, number of hits validated and number of confirmed lead compounds (>1.8-fold increase) are indicated.

(legend continued on next page)
signaling co-receptors \(p75^{NTR}\) and paired immunoglobulin-like receptor B (PIR-B). Key components of the glial scar are the chondroitin sulfate proteoglycans (CSPGs), a family of extracellular matrix proteins that inhibit axon outgrowth (Dickendesher et al., 2012; Lang et al., 2014; Shen et al., 2009; Snow et al., 2003).

Preventing extrinsic inhibitory signals has real potential for promoting CNS axon regeneration (Case and Tessier-Lavigne, 2005). In addition, however, complementary approaches aiming to boost axonal growth without targeting a specific inhibitory mechanism also have potential therapeutic impact. Among small molecules reported to be active on axon growth are monastrol, an inhibitor of kinesin-5 (Baas and Matamoros, 2015), and inhibitors of the mevalonate pathway, through which cholesterol and isoprenoids are synthesized from acetyl-coenzyme A (Figure 3A; Pooler et al., 2006). However, the latter compounds were tested at high concentrations that have been found to be cytotoxic in other studies (Butterfield et al., 2003). Moreover, those small molecules that have been shown to be effective in the CNS in vivo, such as cyclic AMP (cAMP)/rolipram, ROCK inhibitor, and polyamines, lack the specificity and potency expected of clinical candidates (Fournier et al., 2003; Nikulina et al., 2004; Cai et al., 1999, 2002). There is therefore a need to explore more broadly ways in which the suboptimal intrinsic growth capacity of adult CNS neurons can be boosted.

Phenotypic cell-based screening provides a powerful, unbiased chemical genetic approach to identifying novel mechanisms underlying a given cellular process. Earlier studies taking this approach to axonal growth used relatively small screening libraries (<2,000 small molecules; Al-Ali et al., 2015; Koprivica et al., 2005; Ma et al., 2010; Usher et al., 2010). We instead developed a high-throughput screen using neurons grown in the presence of myelin inhibitors. The screen identified four active compound classes, of which we focused on the cholesterol-lowering drugs statins. We show that statins are the most potent agents reported to promote growth of mouse and human neurons in culture and show strong regenerative effects when administered to injured CNS neurons in vivo. Statin activity in this system entirely reflects regenerative effects when administered to injured CNS neurons grown in the presence of myelin inhibitors. The screen collections tested and for assay optimization procedures). Approximately 98% of compounds showed no significant effect on neurite outgrowth, while a minority were toxic (Figures 1D and 1E). Initial hits were defined as compounds that increased neurite outgrowth by >30% (initial hit rate, 1.6%; Figure 1E). In repeat assays using five-point 2-fold dilution series, 151 compounds were confirmed to be active. Of these, 91 were reordered from vendors and tested in a nine-point 2-fold dilution series in six replicates. Finally, four compounds that enhanced neurite outgrowth by >80% at their optimal concentration(s) were selected as leads (Figure S1C). One was a hitherto undescribed compound with no identified target. The second lead shows 90% similarity to a class of phosphodiesterase 4 inhibitors (PDE4; Skoumbourdis et al., 2008) and likely acts by

**RESULTS**

**High-Throughput Screening for Small Molecules that Promote Neurite Regeneration**

To identify mechanisms of axonal regeneration, we developed a high-throughput neuron-based assay (Figure 1). We used rodent motor neurons, since drugs screened on primary rat motor neurons subsequently showed efficacy in the clinic (Bordet et al., 2007). For ease of scale-up, mouse embryonic stem cell-derived motor neurons (mES-MNs) expressing an Hb9::GFP reporter (~50% abundance) were employed (ESC; Wichterle et al., 2002). To quantify neurite outgrowth with minimal sampling error, we used fluorescence live imaging of whole wells in 96-well format (typically 300–700 neurons imaged per well) followed by automated image analysis that identified individual neurons and calculated the neurite length for each (Figure 1A).

To mimic the inhibitory cellular environment of the adult CNS, ES-MNs were grown on monolayers of CHO cells expressing MAG (Mukhopadhyay et al., 1994). After 20 hr in culture, neurite outgrowth of ES-MNs on MAG-CHO cells was only 47% ± 2% (mean ± SEM, \(n = 8\)) of that on control CHO cells (Figures 1A and 1B), whereas neuronal survival was only slightly affected (MAG-CHO 92% ± 1% of control; \(p < 0.008\)). Primary embryonic day 12.5 (E12.5) mouse motor neurons showed the same degree of growth inhibition on MAG-CHO cells (Figure 1B). Increasing cAMP levels has been shown to overcome MAG inhibition of axonal growth (Qiu et al., 2002), and the phosphodiesterase inhibitor rolipram promotes regeneration in vivo (Nikulina et al., 2004). Accordingly, forskolin promoted neurite growth in our assay, with a half-maximal effective concentration (EC_{50}) of 0.85 ± 0.08 \(\mu M\) (Figure S1A), as did a combination of forskolin (10 \(\mu M\)) and IBMX (100 \(\mu M\)), which was used as a positive control for the screen (Figure S1B). Our assay therefore faithfully reproduced the activity of compounds known to enhance axonal regeneration in vivo.

We screened a library of 50,401 compounds at a single concentration (10 \(\mu M\)) for their ability to overcome MAG inhibition (Figure 1C; see Experimental Procedures for the compound collections tested and for assay optimization procedures). Approximately 98% of compounds showed no significant effect on neurite outgrowth, while a minority were toxic (Figures 1D and 1E). Initial hits were defined as compounds that increased neurite outgrowth by >30% (initial hit rate, 1.6%; Figure 1E). In repeat assays using five-point 2-fold dilution series, 151 compounds were confirmed to be active. Of these, 91 were reordered from vendors and tested in a nine-point 2-fold dilution series in six replicates. Finally, four compounds that enhanced neurite outgrowth by >80% at their optimal concentration(s) were selected as leads (Figure S1C). One was a hitherto undescribed compound with no identified target. The second lead shows 90% similarity to a class of phosphodiesterase 4 inhibitors (PDE4; Skoumbourdis et al., 2008) and likely acts by
increasing endogenous cAMP levels. The third lead (Figure S1C) bore a close resemblance to a family of kinesin-5 inhibitors, others of which have been reported to trigger axonal outgrowth in culture (Haque et al., 2004). Our open-ended screen was therefore able to detect modulators of known growth-inhibitory pathways. However, we detected no biological activity in this system for either of two small molecules reported to show neurite outgrowth-promoting properties: daidzein (Ma et al., 2010) or erlotinib (Koprivica et al., 2005; Figure S1D). Subsequently, therefore, we focused on the fourth and most potent lead, simvastatin.

**Statins Act Directly on CNS Neurons to Promote Neurite Outgrowth on Multiple Substrata**

Simvastatin is a widely used cholesterol-lowering drug. Six of the other commercially available drugs (cerivastatin, fluvastatin, simvastatin, lovastatin, mevastatin, and pitavastatin) enhanced ES-MN neurite outgrowth on MAG by >2-fold at concentrations ranging from 30 nM to 7 μM (Figure 1F). The remaining three (pravastatin, rosuvastatin, and atorvastatin) showed only weak activity, perhaps because they were designed to be selective for liver cells (Bucket et al., 2000; McTaggart et al., 2001). Cerivastatin was the most potent and induced a ~3-fold increase in neurite outgrowth with an EC₅₀ of 33.6 ± 2.2 nM (mean ± SEM; n = 6), completely overcoming MAG inhibition (Figure 3B). Cerivastatin was 380-fold more potent in this system than the benchmark ROCK inhibitor Y-27632 (Figure 1G) (Fournier et al., 2003). We therefore used cerivastatin as a tool compound to study the mechanism of action of statins on neurite outgrowth.

To exclude the possibility that statins act indirectly through other cell types in the culture, we showed that MAG protein levels in MAG-CHO cells were not changed (Figures S2A and S2B) and that the effects of cerivastatin on fluorescence-activated cell sorting (FACS)-purified ES-MNs were similar to those on mixed cultures, increasing outgrowth 2.5-fold with an EC₅₀ of 94.7 ± 6.9 nM (Figure 2A). Primary motor neurons from E12.5 ventral spinal cord from Hb9::GFP mice responded similarly to statins, though at slightly higher doses than for ES-MNs (Figures 2A and 2B). Lastly, both primary hippocampal (HN) and primary cerebellar granule neurons (CGN) respond to statins (Figure 2A). Thus, statins act directly on all neuronal populations tested.

To determine whether statins only inhibit a MAG-specific signaling mechanism (Filbin, 2003) or act more broadly on regeneration, we administered cerivastatin to ES-MNs grown on different substrata. Cerivastatin was also effective when cultured on both inhibitory and permissive substrata, polyornithine-laminin and control CHO cells (which showed higher basal neurite outgrowth), we observed a significant increase in outgrowth (Figures 2C and 2D). Although cerivastatin has a significant effect on neurite initiation, its strongest dose-dependent effects are on axon elongation (Figures S2C–S2F). Therefore, statins act as potent general promoters of neurite outgrowth on all substrata tested, suggesting that basal levels of signaling through the mevalonate pathway are regulated by statins.
pathway provide a brake on axonal growth even on the most supportive substrata.

**Statins Promote Neurite Outgrowth by Inhibiting 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase**

The cognate target of all statins is the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGCR), which catalyzes a rate-limiting upstream step in cholesterol biosynthesis (Figure 3A). If statins stimulate axonal growth by inhibiting HMGCR, adding back the enzyme product mevalonate (which is cell permeable) in the presence of statin should restore inhibition. Mevalonate alone had no effect on neurite growth on either control or MAG-CHO monolayers, while addition of increasing concentrations of mevalonate in the presence of a fixed 500 nM concentration of statin, itself capable of completely overrunning myelin inhibition, fully reconstituted inhibition by MAG (Figure 3B). Therefore, HMGCR inhibition accounts for all neurite-promoting actions of cerivastatin, and endogenous levels of mevalonate are not limiting for full inhibition by MAG. Nevertheless, inhibiting this pathway necessarily leads to reductions in the levels of cholesterol, which could be damaging in the CNS, where it plays multiple roles in synapse function. We therefore sought a means to mimic the biological effects of statins on axon growth without affecting cholesterol.

**Inhibition of Protein Prenylation Accounts for the Statin-Induced Increase in Axonal Growth**

Downstream of HMGCR, multiple branches of the mevalonate pathway lead to the synthesis of isoprenoids involved in the synthesis of cholesterol as well as lipid intermediates required for post-translational protein prenylation (Figure 3A; van der Most et al., 2009; Corsini et al., 1999). We examined the role of each in the inhibition of neurite outgrowth. The last intermediate common to all the branches is farnesyl pyrophosphate (Fpp). Using add-back experiments (Wasko et al., 2011), we confirmed that Fpp prevents growth enhancement by cerivastatin (Figure 3C). In contrast, neither coenzyme Q4 or Q10 (from the left-hand branch in Figure 3A) nor high-density lipoprotein (HDL)- or low-density lipoprotein (LDL), which affect cholesterol synthesis, reconstituted the growth inhibition (Figures S3B–S3E), demonstrating that it is not necessary to block cholesterol or coenzyme Q synthesis to enhance neurite outgrowth.

However, application of Gppp to ES-MN cultures treated with cerivastatin completely restored inhibition by MAG (Figures 3C and D). The strong, selective effects of Fpp and Gppp pointed to inhibition of protein prenylation as the principal mode of statin action in our system. To confirm the involvement of prenylation enzymes, we first tested small-molecule inhibitors GGTI-298 and 2153) promote neurite outgrowth synergistically, since the value for increased length above baseline is more than additive (mean ± SEM; n = 6, p < 0.001). Note that a combination of both inhibitors drives neurite outgrowth to levels comparable to those of vehicle-treated neurons on non-inhibitory control CHO cells (unfilled bar).
Figure A: Images showing proximal (~1.2 mm) and distal (~1.8 mm) regions of the axons treated with different conditions:
- **Saline**
- **Cerivastatin**
- **GGTi + FTI**

Figure B: Graph showing the mean gray value of CTB-labeled axons (A.U.) at different distances from the lesion site (μm) for different treatments:
- **Saline**
- **Cerivastatin**
- **GGTi** 50 μM
- **FTI** 50 μM
- **GGTi** 100 μM
- **FTI** 100 μM
- **GGTi + FTI** 50 μM each

Figure C: Graph showing the average of the 5 longest axons (μm) for different treatments and concentrations:
- **Saline**
- **Cerivastatin** 50 μM
- **FTI** 50 μM
- **GGTi** 100 μM
- **GGTi + FTI** 50 μM each

Figure D: Images of Tuj1+ cells/section for different treatments and conditions:
- **No crush**
- **Crush**
- **Saline**
- **Cerivastatin**
- **GGTi + FTI**

Figure E: Graph showing the number of Tuj1+ cells/section for different treatments:
- **Saline**
- **Cerivastatin**
- **GGTi + FTI**
and FTI-277, each of which is incompletely selective (see Figure S3A for in vitro half-maximal inhibitory concentration \((IC_{50})\) values for both PGGT-1 and PFT). Both promoted neurite growth in a dose-dependent manner (GGTI-298: \(EC_{50} = 2.6 \pm 0.21 \mu M, n = 7\); FTI-277: \(5.6 \pm 0.21 \mu M, n = 6\); Figure 3E). To determine whether one or both of the two prenylation pathways was involved, inhibitors with greater specificity for their respective targets, GGTI-2417 and FTI-2153, were tested (Figure S3A). Tested alone, GGTI-2417 had no significant effect \((p = 0.266)\) whereas FTI-2153 led to a 1.7-fold increase in neurite outgrowth \((p < 0.001; \text{Figure 3F})\). Strikingly, though, their actions were strongly synergistic \((p < 0.001)\) for a synergistic as opposed to additive effect; Bliss model). The combined inhibition of PGGT-1 and PFT was sufficient to induce a 3-fold increase in axon outgrowth on MAG over the 20-hr culture period (Figure 3F). The resulting growth on MAG was similar to that on control CHO cells. Therefore, inhibition of dual protein prenylation pathways can stimulate axonal growth without inhibiting cholesterol synthesis (Figure 6F).

**Statins and Prenylation Inhibitors Promote CNS Axon Regeneration In Vivo**

To explore the role of this mechanism in mature CNS neurons in vivo, we used the optic nerve crush model, in which compounds applied into the eye gain direct access to injured retinal ganglion cells (RGCs) (Figure 4). The optic nerve of 6- to 8-week-old mice was injured by pinching with forceps, and immediately afterward, a total volume of 2 \(\mu L\) saline, 25 \(\mu M\) cerivastatin, 50 or 100 \(\mu M\) GGTI-2417 (GGTI), 50 or 100 \(\mu M\) FTI-2153 (FTI), or a combination of 50 \(\mu M\) GGTI and 50 \(\mu M\) FTI was intravitreally injected into the peripheral zone of the eye. The final total (free + bound) concentrations of these compounds in the vitreous was estimated to be in the micromolar range. At 14 days post-injury, live regenerating axons were labeled by anterograde tracing using labeled cholera toxin \(\beta\)-subunit and fixed 3 days later (Figure 4A). We confirmed that the same axons expressed GAP-43, a marker of regenerating axons (Figure S4; Kопrivica et al., 2005). Mice treated with saline showed few regenerating axons distal to the lesion site (Figure 4A). However, following treatment with cerivastatin, robust regrowth of RGC axons was observed (average of five longest axons: saline = 371 ± 64 \(\mu m\) versus cerivastatin = 1,618 ± 137 \(\mu m, p < 0.01; \text{Figures 4A and 4C})\). To provide a more complete measure, we quantified cholera toxin B subunit (CTB) labeling on serial sections of optic nerves at different distances from the lesion (Figure 4B). There was a highly significant effect \((p < 0.01)\) of cerivastatin as compared to saline control at all positions in the nerve. Following nerve crush in 24-week-old mice, statin treatment also conferred benefit (not shown).

We next asked whether, as in vitro, prenylation inhibitors could mimic the beneficial effect of statins. Effects of the combination of 50 \(\mu M\) GGTI-2417 and 50 \(\mu M\) FTI-2153 were indistinguishable from that of cerivastatin, at all distances from the lesion (Figures 4A–4C). Strikingly, neither of the inhibitors used singly had a robust effect on optic nerve regeneration, making the synergistic effect of the combination highly significant \((p < 0.01; \text{Figure 4C})\). One possible explanation of the increased axonal growth might have been protection of RGCs from injury-induced death, which we determined to be 77% after 2 weeks in saline-treated mice (Figures 4D and 4E). However, no neuroprotective effect of either cerivastatin or a combination of GGTI + FTI was observed (Figures 4D and 4E), suggesting that both agents act primarily to enhance growth of regenerating CNS axons in vivo.

**Significance of the Mevalonate and Prenylation Pathway for Axonal Degeneration and Regeneration in Human Patients with ALS**

All experiments thus far had used mouse models. We therefore tested inhibitors on two systems of human stem cell-derived motor neurons: the “gold-standard” ES-derived motor neurons expressing an Hb9::GFP reporter as well as human induced pluripotent stem cell-derived motor neuron (hiPSC-MNs) derived from ALS patients and controls (Amoroso et al., 2013, Boulting et al., 2011, Maury et al., 2015). Human neurons grew well on control CHO cells and, like their mouse counterparts, showed clear growth inhibition on a MAG-CHO cell monolayer (Figure 5A) but little reduction in survival (values for MAG-CHO were 89%–92% of those on control CHO cells). Addition of cerivastatin led to a dose-dependent, 6-fold increase in axon length over the 20-hr culture period (Figures 5A and 5B). The fold-increase of neurite length was even stronger than on mouse motor neurons, though the \(EC_{50}\) was higher \((390 ± 56 nM; n = 6\). Hb9::GFP motor neurons in mixed cultures \((~30%\) abundance) or purified by FACS responded in a comparable manner to cerivastatin (Figure 5B). Introduction of an ALS-causing point mutation into the SOD1 gene of the same ES line only modestly reduced the ES-MN response to statins (Figure 5C). To directly confirm that statins can affect human ALS motor neurons, we generated hiPSC-MNs from the 007 human induced pluripotent stem cell (hiPSC) line derived from a patient with an SOD1\(^{AV}\) point mutation, as well as an isogenic control line in which the mutation had been corrected (Figure 5D). Cerivastatin strongly promoted neurite outgrowth from both, and once again, ALS motor neurons...
responded similarly to their control counterparts. Effects on survival were minor (<50% increase in the presence of statins as compared to >600% for neurite outgrowth).

Lastly, we tested the prenylation inhibitors GGTI-2417 and FTI-2153, alone and in combination, on hiPS-MNs from the 18c healthy control line (Lamas et al., 2014) grown on MAG-CHO cells (Figure 5E). Their synergistic effects were striking; neither compound tested alone significantly stimulated axon growth, but in combination, they triggered a 3-fold increase over the first 20 hr of culture.

To determine whether protein prenylation might also regulate growth in adult human motor neurons in vivo, we turned to a published microarray analysis of lumbar motor neurons that were laser captured from ALS patients who had died of the bulbar form of the disease, meaning that many motor neurons still survived at the less-affected lumbar levels (Rabin et al., 2010). The only other two mevalonate pathway genes to show a degree of correlation with age were ACAT1 and MVD (Figure 6A). However, the difference in expression levels between early- and late-onset ALS was low (<1.5-fold for both genes), suggesting that the functional impact was not significant.

**DISCUSSION**

Our understanding of the cell-intrinsic mechanisms that regulate neurite growth remains incomplete. In particular, there is a critical need for a means by which to enhance axonal regeneration in human patients. By conducting an extensive screen for small molecules able to overcome myelin inhibition, we found that statins are the most potent neurite growth-promoting agents yet reported, both for rodent and human neurons. Moreover, comparable effects on axonal regeneration can be generated, without blocking cholesterol synthesis, by inhibiting both branches of the protein prenylation pathway, either in vitro or in vivo. In all neurons examined, we found that endogenous levels of prenylation are maintained at a level that constitutes a brake on axonal growth, and expression data suggest this may contribute to lack of compensatory sprouting in early-onset ALS patients. Targeting the mevalonate-prenylation pathway may therefore allow both preventive and reparative approaches in human patients with CNS trauma or neurodegenerative disease.

Other screens reported to date have used different cellular models and ~20-fold-smaller screening libraries (Al-Ali et al., 2013). However, our comprehensive unbiased screen identified mevalonate inhibition as a major mechanism to significantly enhance neurite growth.
### A

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>p value correlation</th>
<th>p value early vs late</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT1</td>
<td>Acetoacetyl-CoA transferase ACAT1</td>
<td>0.008</td>
<td>0.05</td>
</tr>
<tr>
<td>ACAT2</td>
<td>Acetoacetyl-CoA transferase ACAT2</td>
<td>0.653</td>
<td>0.56</td>
</tr>
<tr>
<td>HMGCS1</td>
<td>HMG-CoA synthase1</td>
<td>0.884</td>
<td>0.91</td>
</tr>
<tr>
<td>HMGCS2</td>
<td>HMG-CoA synthase2</td>
<td>0.372</td>
<td>0.23</td>
</tr>
<tr>
<td>HMGCR</td>
<td>HMG-CoA reductase</td>
<td>0.915</td>
<td>0.83</td>
</tr>
<tr>
<td>MVK</td>
<td>Mevalonate kinase</td>
<td>0.220</td>
<td>0.42</td>
</tr>
<tr>
<td>PMVK</td>
<td>Phosphomevalonate kinase</td>
<td>0.344</td>
<td>0.29</td>
</tr>
<tr>
<td>MVD</td>
<td>Mevalonate-5-pyrophosphate decarboxylase</td>
<td>0.030</td>
<td>0.05</td>
</tr>
<tr>
<td>IDI1</td>
<td>Isopentenyl-diphosphate isomerase 1</td>
<td>0.751</td>
<td>0.80</td>
</tr>
<tr>
<td>FDPs</td>
<td>Farnesyl diphosphate synthase</td>
<td>0.906</td>
<td>0.86</td>
</tr>
<tr>
<td>FNTA</td>
<td>Farnesyl transferase alpha subunit</td>
<td>0.213</td>
<td>0.12</td>
</tr>
<tr>
<td>FNTB</td>
<td>Farnesyl transferase beta subunit</td>
<td>0.985</td>
<td>0.96</td>
</tr>
<tr>
<td>GGPS1</td>
<td>Geranyleranyl diphosphate synthase</td>
<td>0.139</td>
<td>0.19</td>
</tr>
<tr>
<td>PGGT1B</td>
<td>Geranyleranyl transferase beta subunit</td>
<td>0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>RABGGTA</td>
<td>Rab geranyleranyl transferase, alpha subunit</td>
<td>0.534</td>
<td>0.36</td>
</tr>
<tr>
<td>RABGGTB</td>
<td>Rab geranyleranyltransferase, beta subunit</td>
<td>0.093</td>
<td>0.31</td>
</tr>
</tbody>
</table>

### B

![Graph](image1)

### C

![Graph](image2)

### D

![Graph](image3)

### E

![Graph](image4)

---

**F**

untreated  
Prenylation brake

+GGTI  

+FTI  
Early-onset ALS

Statin  
or  
+FTI  
+GGTI  
Late-onset ALS

(legend on next page)
Figure 6. Levels of PGGT1B Are Selectively Elevated in Motor Neurons of Patients with Early-Onset ALS as Compared to Late-Onset Patients or Healthy Controls  
(A) Based on the raw data of Rabin et al. (2010), for each enzyme in the different branches of the mevalonate pathway, we calculated the significance of the overall difference between ALS and control patients (p value correlation) and the difference between the values in early-onset (<60 years) and late-onset (>60 years) ALS patients (p value early versus late). Only PGGT1B, which encodes the \( \beta \) subunit of PGGT-1, showed a significant correlation with age of onset as well as a significant fold-difference in expression levels between early– and late-onset cases.  
(B–E) Scatterplots of expression levels of individual genes analyzed in this study in ALS patients (green) and controls (red). Whereas ALS and control values were indistinguishable for the first three genes (with some scatter for HMGCR), early-onset patients expressed high levels of PGGT1B that decreased progressively with increasing age. No similar change was seen in healthy controls over the same age range or in dorsal spinal neurons from the same ALS patients (p value early versus late). Only

likely reflecting these differences, in our system, we could not detect activity of either daidzein, which has already been reported to work only when neurons are primed by pre-exposure to drug (Ma et al., 2010), or erlotinib (Koprivica et al., 2005; Figure S1D). In our hands, despite the specific nature of our screening model, statins were effective on all neurons (both stem cell derived and primary) and all substrata (both inhibitory and permissive) tested. They seem therefore to trigger a general boost in axon growth potential.

The tissue that expresses the highest levels of HMGCR is the brain (Burkhardt et al., 2008). Multiple and often contradictory effects of statins and prenylation inhibitors on cultured neurons have been reported. These include changes in directional growth of axons without any increase in axonal length (Holmberg et al., 2006), protection of axons against excitotoxicity (Posada-Duque et al., 2013), decreased neurite length (Schulz et al., 2004, Kim et al., 2009), or no effect (Fan et al., 2002). The data most comparable to ours are those of Pooler et al. (2006), who report a 40% increase in neurite length of hippocampal neurons following exposure to high concentrations (≥100 \( \mu \)M) of pravastatin or 50 \( \mu \)M GGTI-286, but not FTI-277. But these concentrations, which are at least an order of magnitude above the \( EC_{50} \) were found to be toxic in our study and other studies (Figures 2C, 2D, and 3E; Butterfield et al., 2011). It is unclear whether these disparities are due to specificities of neurons and substrata or the methodology of quantification and cell culture, and in vivo validation is therefore critical. Our finding that a single intravitreal injection of statin or prenylation inhibitors was sufficient to support RGC axonal growth over 2 weeks at levels comparable with many genetic manipulations was highly encouraging. It is reasonable to suppose that the effect will be still greater when the drug can be delivered continuously and in the presence of an agent that prevents the >75% loss of RGCs caused by axotomy and not rescued by cerivastatin.

Inhibition of protein prenylation is considered to play a key role in many biological effects of statins not directly linked to cholesterol (Samuel and Hynds, 2010). Hundreds of intracellular signaling proteins (e.g., small GTP/GDP-binding proteins) may potentially be prenylated, but only a minority of modifications have been specifically identified (Samuel and Hynds, 2010; Charron et al., 2013). Some, like K-Ras, Ras, and H-Ras, are exclusively farnesylated, while others, such as Rac, Rho, and Rap, are geranylgeranylated; lastly, one (RhoB) is modified by both enzymes (Zhang and Casey, 1996). We found both in vitro and in vivo that inhibiting both farnesylation and geranylgeranylation provided the strongest benefit for axonal growth. This may suggest a central role for a doubly modified protein, or it may mean that multiple prenylation substrates are involved. Distinguishing between such possibilities is complicated. First, compensatory prenylation has also been described; when PFT is inhibited by FTIs, K-Ras and N-Ras, but not H-Ras, become geranylated. Second, knockdown or inhibition of Rac1 was shown to mimic the neuroprotective actions of statins on cortical neurons (Posada-Duque et al., 2013), yet a non-prenylatable mutant of Rac1 was recently shown to retain its function in a neurite outgrowth assay (Reddy et al., 2015).

Given the widespread usage of statins, many authors have addressed the question of whether they are protective and/or beneficial in CNS indications. Citing only the most recent meta-analyses for multiple sclerosis, cognition in Alzheimer’s disease, and Parkinson’s disease, the response is unclear (Pihl-Jensen et al., 2015; Chatterjee et al., 2015; Huang et al., 2015), although there are signs that statin use in patients with intracerebral hemorrhage may be associated with improved mortality and functional outcomes (Jung et al., 2015). One reason for inconsistency or lack of effect is the very limited information we have on brain exposure and pharmacodynamics for statins in general. It is generally considered that lipophilic statins like simvastatin lactone cross the blood–brain barrier, whereas atorvastatin, which is relatively less lipophilic, does not (Serajuddin et al., 1991). However, we performed a pharmacokinetic study (not shown) for cerivastatin and simvastatin and found both to have extremely short half-lives in rodents, whether administered centrally or peripherally.

Patients expected to have high endogenous levels of prenylation in their motor neurons, through higher expression of PGGT1B, showed earlier ALS onset, perhaps due to their reduced ability to reinervate denervated muscle fibers (see model in Figure 6F). We did not find a difference in basal outgrowth between the ALS and control hiPS-MNs we analyzed in vitro. However, this was not unexpected, since hiPS-MNs are embryonic in phenotype and have not been exposed to the multiple epigenetic influences that may lead to changes in gene

2015; Ma et al., 2010; Usher et al., 2010; Koprivica et al., 2005). Likely reflecting these differences, in our system, we could not detect activity of either daidzein, which has already been reported to work only when neurons are primed by pre-exposure to drug (Ma et al., 2010), or erlotinib (Koprivica et al., 2005; Figure S1D). In our hands, despite the specific nature of our screening model, statins were effective on all neurons (both stem cell derived and primary) and all substrata (both inhibitory and permissive) tested. They seem therefore to trigger a general boost in axon growth potential.

The tissue that expresses the highest levels of HMGCR is the brain (Burkhardt et al., 2008). Multiple and often contradictory effects of statins and prenylation inhibitors on cultured neurons have been reported. These include changes in directional growth of axons without any increase in axonal length (Holmberg et al., 2006), protection of axons against excitotoxicity (Posada-Duque et al., 2013), decreased neurite length (Schulz et al., 2004, Kim et al., 2009), or no effect (Fan et al., 2002). The data most comparable to ours are those of Pooler et al. (2006), who report a 40% increase in neurite length of hippocampal neurons following exposure to high concentrations (≥100 \( \mu \)M) of pravastatin or 50 \( \mu \)M GGTI-286, but not FTI-277. But these concentrations, which are at least an order of magnitude above the \( EC_{50} \) were found to be toxic in our study and other studies (Figures 2C, 2D, and 3E; Butterfield et al., 2011). It is unclear whether these disparities are due to specificities of neurons and substrata or the methodology of quantification and cell culture, and in vivo validation is therefore critical. Our finding that a single intravitreal injection of statin or prenylation inhibitors was sufficient to support RGC axonal growth over 2 weeks at levels comparable with many genetic manipulations was highly encouraging. It is reasonable to suppose that the effect will be still greater when the drug can be delivered continuously and in the presence of an agent that prevents the >75% loss of RGCs caused by axotomy and not rescued by cerivastatin.

Inhibition of protein prenylation is considered to play a key role in many biological effects of statins not directly linked to cholesterol (Samuel and Hynds, 2010). Hundreds of intracellular signaling proteins (e.g., small GTP/GDP-binding proteins) may potentially be prenylated, but only a minority of modifications have been specifically identified (Samuel and Hynds, 2010; Charron et al., 2013). Some, like K-Ras, Ras, and H-Ras, are exclusively farnesylated, while others, such as Rac, Rho, and Rap, are geranylgeranylated; lastly, one (RhoB) is modified by both enzymes (Zhang and Casey, 1996). We found both in vitro and in vivo that inhibiting both farnesylation and geranylgeranylation provided the strongest benefit for axonal growth. This may suggest a central role for a doubly modified protein, or it may mean that multiple prenylation substrates are involved. Distinguishing between such possibilities is complicated. First, compensatory prenylation has also been described; when PFT is inhibited by FTIs, K-Ras and N-Ras, but not H-Ras, become geranylated. Second, knockdown or inhibition of Rac1 was shown to mimic the neuroprotective actions of statins on cortical neurons (Posada-Duque et al., 2013), yet a non-prenylatable mutant of Rac1 was recently shown to retain its function in a neurite outgrowth assay (Reddy et al., 2015).

Given the widespread usage of statins, many authors have addressed the question of whether they are protective and/or beneficial in CNS indications. Citing only the most recent meta-analyses for multiple sclerosis, cognition in Alzheimer’s disease, and Parkinson’s disease, the response is unclear (Pihl-Jensen et al., 2015; Chatterjee et al., 2015; Huang et al., 2015), although there are signs that statin use in patients with intracerebral hemorrhage may be associated with improved mortality and functional outcomes (Jung et al., 2015). One reason for inconsistency or lack of effect is the very limited information we have on brain exposure and pharmacodynamics for statins in general. It is generally considered that lipophilic statins like simvastatin lactone cross the blood–brain barrier, whereas atorvastatin, which is relatively less lipophilic, does not (Serajuddin et al., 1991). However, we performed a pharmacokinetic study (not shown) for cerivastatin and simvastatin and found both to have extremely short half-lives in rodents, whether administered centrally or peripherally.

Patients expected to have high endogenous levels of prenylation in their motor neurons, through higher expression of PGGT1B, showed earlier ALS onset, perhaps due to their reduced ability to reinervate denervated muscle fibers (see model in Figure 6F). We did not find a difference in basal outgrowth between the ALS and control hiPS-MNs we analyzed in vitro. However, this was not unexpected, since hiPS-MNs are embryonic in phenotype and have not been exposed to the multiple epigenetic influences that may lead to changes in gene
expression in adult human neurons. Based on our data, one hypothesis would be that patients taking statins would show a greater propensity for compensatory axonal sprouting, meaning that with a given risk from other causes, they would either develop ALS later or not at all. Although earlier meta-analyses were not conclusive (Zheng et al., 2013), a recent large study (722 ALS patients and 2,268 controls) showed evidence for a protective effect of statins against the onset of ALS (Seelen et al., 2014). In accordance with our hypothesis, the major contributor to the improved odds ratio was simvastatin, which is considered the most brain penetrant of the statins.

Our data show that neurons in multiple contexts maintain endogenous levels of prenylation that restrict their maximum growth capacity. In the adult CNS, exuberant sprouting might lead to circuit dysfunction and behavioral abnormalities. However, it is less clear why embryonic neurons growing on permissive substrata would need a brake on their growth. One intriguing possibility is raised by studies of extracellular signaling molecules such as semaphorins, which control not only the direction but also the timing of axon growth to specific territories during embryogenesis (Huber et al., 2005). Therefore, activity of the mevalonate-prenylation pathway may potentially govern the establishment of neural circuits in a cell-intrinsic manner.

Overall, our data support the idea that the mevalonate-prenylation pathway represents a target for therapeutic approaches to currently incurable diseases and trauma of the CNS. Although the small molecules currently available to target the pathway have limitations in terms of bioavailability and rare on-target toxicity (Cziraky et al., 2013), the exceptional potency of the effects they engender in model systems suggests that therapeutic strategies to inhibit prenylation should be pursued for currently incurable diseases such as ALS.

EXPERIMENTAL PROCEDURES

mES-MN and hiPS-MN Differentiation

Mouse ESCs carrying the Hb9::GFP transgene were differentiated into motor neurons as described previously (Vichterle et al., 2002). Human ES-MNs and hiPS-MNs from healthy control and ALS patients were differentiated as described in Lamas et al. (2014) (Amoroso et al., 2013; Boulting et al., 2011; Maury et al., 2015). Briefly, human ESC colonies were cultured in ESC medium for another 10 days in neural differentiation medium (Neurobasal medium supplemented with 10 ng/ml glial cell line-derived neurotrophic factor (GDNF), and 10 ng/ml brain-derived neurotrophic factor (BDNF) for 4 days before they were switched to neural induction medium (DMEM/F12 medium with 200 ng/ml retinoic acid and 2% B-27 supplement) and the intravitreal injection in 6- to 8-week-old mice were performed as described previously (Park et al., 2008). After the nerve crush, 2 μl of saline or cerivastatin, GGTI-2417, FTI-2153 or combinations of GGTI-2417 and FTI-2153 at different concentrations was intraocularly injected into the peripheral retina. Each compound (2 μl) is diluted 1:10 in the vitreous based on the fact that the vitreous volume is 20 μl (Saszik et al., 2002). 14 days after injury, 2 μl cholecystokinin (cubulin subunit coupled to Alexa Fluor 555 (CTB-555) (1 μg/μl, Life Technologies) was intraocularly injected into the retina. 3 days after CTB injection, the eye with optic nerve was dissected out, post-fixed in 4% PFA overnight, and cryoprotected in 30% sucrose in PBS. Eye and optic nerves were separately frozen on dry ice, and 10-μm cryosections were prepared for axon regeneration and RGC survival analysis.

Automated Quantification of Axon Regeneration and RGC Survival

To quantify CTB-labeled regenerating axons, three cryosections of ~2 mm optic nerve per animal were washed with PBS and mounted with an aqueous mounting medium. CTB-labeled regenerating axons were imaged in grayscale using a constant exposure time. In each section, ImageJ provided a mean gray value of CTB-labeled axons in each 200-μm optic nerve column from the lesion
site after removing the value on background (non-tissue area) in the same image. The average of mean gray value of regenerating axons in each 200-µm optic nerve column was obtained from three sections per animal. Each condition was tested with three to six animals (n = 3–6). To obtain length of the five longest axons, the distance of the five longest axon tips from the injury site was measured with ImageJ in each section. The average of the five longest axons was obtained from three sections per animal. Each condition was tested with three to six animals (n = 3–6). To examine RGC survival, the 60th–80th sections of ~150 sagittal cryosections of the whole retina per animal were immunostained with anti-βIII tubulin antibody. Five sections among them were selected randomly for the analysis. After counting the number of TuJ1+ cells in all five sections per animal, the average number of β-III tubulin+ cells in the retina per animal was obtained, and it was pursued repeatedly with three animals in the same condition (n = 3).

Analysis of Gene Expression Data from Postmortem ALS

The expression data were generated on the Affymetrix HuEx_1.0_st_v2 chip and normalized using the robust multi-array average (RMA) method. To test how the expression levels of a gene of interest vary with age (at death/sample collection) in ALS patients or healthy controls, a linear model was fitted between the log-intensity values and the age at sample collection. Disease duration did not vary significantly among the ALS patients, and the age at onset and age at death were highly correlated (data not shown). The R2 (unadjusted) and p values of the coefficient for age in this model are reported in Figure 6A (column 3). To test whether the genes of interest are expressed differentially between late-onset and early-onset ALS patients, a linear model was fit between the log-intensity values and the ALS categorization (early, late, or control). The p values of the coefficients in this fit are reported in Figure 6A (column 4). Since the above analyses were done on genes comprising the mevalonate pathway (~20 genes) only, we report only the unadjusted p values. All analyses were done in the statistical language R.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.013.

AUTHOR CONTRIBUTIONS

H.L. conducted experiments, analyzed data, and wrote the manuscript. T.K. performed and analyzed optical nerve crush experiments and wrote the manuscript. D.O. and R.H. analyzed gene expression data. E.N. performed experiments with hippocampal neurons and analyzed optic nerve crush experiments. J.H. performed optic nerve crush experiments. W.S.Y. provided support for the compound libraries. E.R.L., N.J.L., M.W.A., G.F.C., and H.W. provided ES or hiPS cell-derived motor neurons. S.S. provided specific prenylation inhibitors and contributed to the writing of the manuscript. M.T.F. coordinated research efforts and provided the CHO cells. B.S. coordinated research efforts and provided the compound libraries. M.T.F. contributed to the writing of the manuscript. C.E.H. coordinated research efforts, supervised research work and data analysis, and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Samahe Fageiry and Cyndel Vollmer for help with the culture model, Kevin Eggan and collaborators for kindly providing Hb9::GFP human ESC reporter lines, and Chuck Karan and members of the High-Throughput Screening and Chemistry Shared Facility for support and technical guidance. We thank the He lab (Harvard) for training in optic in grape. We are grateful to members of the Project A.L.S., Henderson, Wichterle, and Stockwell laboratories at Columbia University for much help and continuous critical discussion. We thank our Biogen colleagues Alex McCampbell, Sha Mi, and Richard Ransohoff for critical reading of the manuscript. This work received invaluable support from the New York State Spinal Cord Injury Research Board (NYS-SCIIRB C020923), P2ALS, Target ALS, the Tow Foundation, the SMA Foundation, Project A.L.S., NYSTEM (C026715), The Helmley Charitable Trust, NINDS R01-NS056422 and NS072428, and the Columbia MD-PhD program. Brent R. Stockwell is an Early Career Scientist of the Howard Hughes Medical Institute, and this research was additionally funded by the NIH (SR01CA097061 and R01CA161061, to B.S.) and New York State Stem Cell Science (C026715).

This article is dedicated to the memory of our dear friend and colleague Marie T. Filbin, a trailblazer in the field of axonal regeneration.

Received: October 20, 2015
Revised: January 31, 2016
Accepted: May 28, 2016
Published: June 30, 2016

REFERENCES


556 Cell Reports 76, 545–558, July 12, 2016


Ma, T.C., Campana, A., Lange, P.S., Lee, H.H., Banerjee, K., Bryson, J.B., Ma-hishi, L., Alam, S., Giger, R.J., Barnes, S., et al. (2010). A large-scale chemical screen for regulators of the arginase 1 promoter identifies the isoform-selective diazoxide clinically approved small molecule that can promote neuronal protection or regeneration via a cAMP-independent pathway. J. Neurosci. 30, 739–748.


