Review

# Cholesterol synthesis inhibition or depletion in axon regeneration

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## Abstract

Cholesterol is biosynthesized by all animal cells. Beyond its metabolic role in steroidogenesis, it is enriched in the plasma membrane where it has key structural and regulatory functions. Cholesterol is thus presumably important for post-injury axon regrowth, and this notion is supported by studies showing that impairment of local cholesterol reutilization impeded regeneration. However, several studies have also shown that statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, are enhancers of axon regeneration, presumably acting through an attenuation of the mevalonate isoprenoid pathway and consequent reduction in protein prenylation. Several recent reports have now shown that cholesterol depletion, as well as inhibition of cholesterol synthesis *per se*, enhances axon regeneration. Here, I discussed these findings and propose some possible underlying mechanisms. The latter would include possible disruptions to axon growth inhibitor signaling by lipid raft-localized receptors, as well as other yet unclear neuronal survival signaling process enhanced by cholesterol lowering or depletion.

**Key Words:** axon regeneration; cholesterol; 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase); lipid raft; methyl-β-cyclodextrin; Nogo receptor; prominin-1; RhoA; statins

## Introduction

Cholesterol is a key metabolite produced in all animal cells and is a precursor to a plethora of steroidal molecules, such as steroid hormones (Miller, 2017). It is also a membrane lipid component and a key determinant of membrane fluidity (Subczynski et al., 2017). Particularly enriched in the plasma membrane, it is important for the structure and function of plasma membrane lipid rafts (Levental et al., 2020; Sviridov et al., 2020), which are cell surface microdomains important for protein trafficking, targeting and signal transduction. Among mammalian organs, the brain contains the largest amount of cholesterol, where it is critical for processes such as myelination (Saher and Stumpf, 2015). In the central nervous system (CNS), adequate cholesterol biosynthesis and delivery in neurons is likely to be indispensable for neuronal structure and function. In the autosomal recessive Niemann-Pick disease type C (NPC) (Hammond et al., 2019), for example, defects in intracellular cholesterol trafficking results in neurodegeneration. Even heterozygous mutations of NPC gene that do not cause disease may be associated with late-onset neurodegenerative disorders (Schneider et al., 2019).

In adult mammals, axon regeneration by neurons in the peripheral nervous system occurs more readily compared to those in the CNS upon injury. This is due at least partly to the presence of inhibitory factors in the adult CNS myelin (Yiu and He, 2006; Uyeda and Muramatsu, 2020) and extracellular matrix (Quraishe et al., 2018). Axon regeneration upon injury by both peripheral and central neurons requires formation and extension of functional growth cones and means of driving the extension of growing axon tips (Rodemer et al., 2020). This would necessitate specific trafficking and targeting of proteins and lipids to the growing axon tip. Some lipids, such as phosphatidylcholine, can be synthesized at the axon (Vance et al., 1991), but cholesterol needs to be transported to the axon from the neuronal soma (Vance et al., 2000). Early evidence based on compartmented cultures of rat sympathetic neurons *in vitro* indicated that neuronal cholesterol synthesis inhibition without any exogenous lipid supply impairs axon growth (de Chaves et al., 1997). *In vivo*, complete nerve repair would also require axonal remyelination. In the CNS, oligodendrocytes elevate their cholesterol levels to facilitate the synthesis of new myelin membranes (Saher and Simons, 2010). In this regard, a functional complementary screening for pro-regenerative factors of olfactory ensheathing cells have identified that *SCARB2* (Roet et al., 2013), which encodes the lysosomal integral membrane protein 2, works in parallel with neuronal NPCs in lysosomal cholesterol export (Heybrock et al., 2019). Axon regeneration was in fact shown to be more sensitive than myelination to manipulations that impaired local cholesterol reutilization after rat sciatic nerve crush (Goodrum et al., 2000).

While all the above attests to the importance of cholesterol in axonal outgrowth and regeneration, there are also findings that suggest that a reduction of cholesterol levels may promote axon regeneration post-injury in both the peripheral nerves and the CNS. Upon rat sciatic nerve crushes, cholesterol synthesis is drastically downregulated post-injury and surprisingly also during nerve regeneration (Goodrum, 1990). Inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 2.3. 3.10), the rate-determining enzyme of cholesterol biosynthesis, promoted neurite outgrowth of rat embryonic cortex explants or postnatal spinal cord explants even on an axon growth inhibitory myelin substrate (Holmberg et al., 2006). Some recent reports have now reaffirmed the notion that axon regeneration could indeed be promoted by cholesterol depletion or cholesterol synthesis inhibition. A search of PubMed (https://pubmed.ncbi.nlm.nih.gov/)

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with the key words "cholesterol" and "axon regeneration" uncovered these and related earlier reports (**Table 1**). We shall first look at these results before pondering on the possible underlying mechanisms.

**Table 1** | **A tabulated summary of studies indicating that cholesterol synthesis inhibition or cholesterol depletion could enhance axon regeneration**

| Study | Experimental system/ model | Methods of cholesterol level manipulation |
|---|---|---|
| Holmberg et al., 2006 | Rat embryonic cortex explants or postnatal spinal cord explants | Simvastatin |
| Tassew et al., 2014 | Chick retinal explants, rat spinal cord compression model | Methyl-β-cyclodextrin (MβCD), Blocking Neogenin lipid raft association with a Ig-like domain peptide |
| Whitlon et al., 2015 | Dissociated mouse spiral ganglia | Cerivastatin |
| Li et al., 2016 | Mouse embryonic stem cell-derived motor neurons | Simvastatin |
| Roselló-Busquets et al., 2019 | Dissociated hippocampal neurons, organotypic slice cultures, sciatic nerve axotomy | MβCD, Nystatin or Cholesterol oxidase |
| Roselló-Busquets et al., 2020 | Hippocampal explants | Nystatin |
| Shabanzadeh et al., 2021 | Chick retinal explant, rat optic nerve crush | Lovostatin, geranylgeranyl pyrophosphate, Δ-7-sterol reductase inhibitors AY9944, BM15766 and silencing of Δ-7-sterol reductase expression |

## Evidence for the Promotion of Axon Regeneration by Cholesterol Synthesis Inhibition

One of the first indications that cholesterol synthesis inhibition may promote axon regeneration came from drug screening data indicating that statins, a class of HMG-CoA reductase inhibitor, promoted neurite outgrowth and axon regeneration (Whitlon et al., 2015; Li et al., 2016). A high content screen of 440 compounds from the NIH Clinical Collection using dissociated mouse spiral ganglia as a neurite outgrowth model has identified a single lead in cerivastatin (CAS 145599-86-6) (Whitlon et al., 2015). In a larger high-throughput screen using mouse embryonic stem cell-derived motor neurons of 50,401 compounds, simvastatin (CAS 79902-63-9) was found to be the most potent lead (Li et al., 2016).

Other modes of lowering or depleting cholesterol levels have the same axon regeneration promoting effect. Monnier's group, for example, has shown that modification of growth cone lipid rafts could promote axon regeneration after spinal cord injury and optic nerve crush (Tassew et al., 2014). This could be achieved using methyl-β-cyclodextrin (MβCD, CAS 128446-36-6) to deplete membrane cholesterol and disrupt cholesterol-rich lipid rafts. A peptide corresponding to the four immunoglobulin-like domains of neogenin (De Vries and Cooper, 2008), which disrupted the interaction between neogenin and the axon growth inhibitor repulsive guidance molecule-a (RGMa) (Hata et al., 2006) at lipid rafts, could also promote axon outgrowth (Tassew et al., 2014). Another recent report by Roselló-Busquets and colleagues showed that Nystatin (CAS 1400-61-9), a cholesterol-binding compound that could extract cholesterol from membranes, promoted axon growth in mice hippocampal explants (Roselló-Busquets et al., 2020). The group also showed that cholesterol depletion by either MβCD, Nystatin or the enzyme cholesterol oxidase (EC 1.1.3.6) enhanced the growth cone morphology of CNS hippocampal and cerebellar external granular layer neurons, as well as the peripheral nervous system dorsal root ganglion (DRG) neurons (Roselló-Busquets et al., 2019), and promoted neurite extension in the latter. Nystatin treatment also enhanced axon regeneration of axotomized E16 hippocampal neurons and organotypic co-cultures. MβCD administered at a non-toxic dose to mice altered the integrity of lipid raft structure in DRG neurons, and increased the expression of the axonal regeneration factor growth-associated protein 43 (Denny, 2006) after sciatic nerve resection, with improved muscle re-innervation and sensory recovery (Roselló-Busquets et al., 2019).

A more recent report by the Monnier group further showed that inhibition of cholesterol synthesis *per se* could promote axon outgrowth by neurons in retinal explants (Shabanzadeh et al., 2021). Interestingly, and contrary to earlier work (Li et al., 2016), the HMG-CoA reductase inhibitor Lovastatin (CAS 75330-75-5) is found to significantly inhibit axonal outgrowth on the permissive substrate laminin, and this inhibition could be reversed by the protein prenylation substrate geranylgeranyl pyrophosphate (GGPP) (CAS 313263-08-0). On the other hand, two inhibitors of Δ-7-sterol reductase (EC 1.3.1.21)) that act at a late-stage of cholesterol synthesis downstream of the HMG-CoA reductase (AY9944 (CAS 366-93-8) and BM15766 (CAS 86621-92-3)), did not alter normal axon growth on laminin compared to control. Axon outgrowth on the inhibitory substrates myelin or RGMa was not affected either way by lovastatin alone, but addition of GGPP enhanced its axon outgrowth promoting effect. Importantly, axon outgrowth on myelin is also enhanced by the late-stage of cholesterol synthesis inhibitors. These results suggest that inhibition of cholesterol synthesis *per se*, not so much the inhibition of protein prenylation, underlies the promotion axon outgrowth on inhibitory substrates. In fact, any axon outgrowth or regeneration promoting effect of statins on non-permissive substrate could be potentially masked by a retardation of axon growth due to protein prenylation inhibition.

Is inhibition of cholesterol synthesis *per se* beyond those steps that generate isoprenoids able to directly promote axon regeneration? The authors showed that treatment of chick brains with either AY9944, lovastatin, or lovastatin + GGPP altered lipid raft formation and neogenin localization. Importantly, either AY9944 or lovastatin in combination with GGPP (but not either of the latter alone) enhanced axonal regeneration after optic nerve injury. Furthermore, siRNA-mediated knockdown of Δ-7-sterol reductase, the final enzyme in the cholesterol synthesis pathway that converts 7-dehydrocholesterol to cholesterol, also promoted axon regeneration. Interestingly, beyond promoting axonal outgrowth, cholesterol inhibition also enhanced retinal ganglion cell survival after optic nerve crush, as well as photoreceptor neuron survival in a mouse model of Retinitis Pigmentosa (Shabanzadeh et al., 2021).

In another study, Lee et al. (2020) showed that prominin-1 (or CD133) (Barzegar Behrooz et al., 2019), a stem cell marker that is developmentally downregulated in mouse DRG neurons, is an intrinsic factor required for axon regeneration. *In vivo*, DRG neurons of *Prom1* knockout (KO) mice have impaired regeneration from sciatic nerve crushes, and *Prom1* KO neurons displayed significant defects in axon regrowth after injury incurred by re-plating in culture. Exogenous over-expression of human *PROM1* in the mice *prom1* KO neurons significantly enhanced axon growth after re-plating, even on an inhibitory substrate of the CNS chondroitin sulfate proteoglycans (CSPGs) (Silver and Silver, 2014). Furthermore, adeno-associated virus-mediated delivery of *prom1* in mice enhanced sciatic nerve regeneration after injury. Transcriptional profiling of embryonic DRG neurons with or without *PROM1* overexpression revealed that *prom1*-differentially expressed genes are nervous system enriched,

but showed only a modest overlap with injury responsive genes. Gene ontology analysis however found that some of the differentially expressed genes are lipid and sterol metabolic genes that were consistently downregulated with *prom1* over-expression. In this regard, prom1 is shown to interact with the type 1 transforming growth factor-β receptor and is required for injury-induced Smad2 phosphorylation and Smad-dependent transcriptional changes, with its downregulation of cholesterol synthesis effected via Smad signaling (Orlova et al., 2016).

## Possible Mechanisms Underlying the Role of Cholesterol Depleting or Cholesterol Synthesis Inhibition in Promoting Axon Regeneration

The recent results summarized above suggest that a reduction in cholesterol promotes axon outgrowth during regeneration, but how does cholesterol lowering produce this effect? A possible mode of action of statin could have been the result of a blanket inhibition of all mevalonate-based isoprenoid synthesis and consequential attenuation of protein prenylation (**Figure 1**). A combined inhibition of the prenylation enzymes farnesyltransferase (EC 2.5. 1.58) and geranylgeranyl transferase type I (EC 2.5.1.59) is known to recapitulated statins' promotion of axon growth on an inhibitory substratum (Li et al., 2016). The well-known axonal growth inhibitory role of the small GTPase RhoA and its effector Rho-associated coiled kinase (ROCK) (Fujita and Yamashita, 2014), which is central to the axonal growth cone growth inhibition or collapsing activity of many myelin-associated inhibitors (Eftekharpour et al., 2017), is dependent on proper C-terminal prenylation of Rho (Reddy et al., 2020). Attenuation of the RhoA-ROCK-mediated growth cone growth inhibitory activity by a reduction in prenylation would thus appear to be particularly relevant for cholesterol lowering-enhanced axonal regrowth in the injured CNS.

However, a major caveat of this interpretation is that inhibition of protein prenylation would be rather indiscriminate. All small GTPases and other molecules that require prenylation for their function will be affected, including some of the pro-regenerative Rac1 (Liu et al., 2018; Scott-Solomon and Kuruvilla, 2020) and Rab family members (Villarroel-Campos et al., 2016). Attesting to this possibility, the results of Shabanzadeh et al. (2021) showed that lovastatin inhibited axon outgrowth on permissive substrates, and did not help outgrowth on myelin unless prenylation defect is first rescued by GGPP. That either Δ-7-sterol reductase inhibitors or its expression silencing were able to promote axon regeneration would suggest that cholesterol reduction *per se* underlies the axon outgrowth promoting effects. This notion would be consistent with the other observations, where direct cholesterol depletion by pharmacological agents such as MβCD and Nystatin (Tassew et al., 2014; Roselló-Busquets et al., 2019, 2020) could also promote axon outgrowth or regeneration. Can this result be reconciled with previous data indicating that statins could enhance axon regeneration? It is notable that the lactone prodrug form of lovastatin has differing effects on axon regeneration compared to regeneration compared to the `activated', hydroxyl acid form (Shabanzadeh et al., 2021). The former significantly promoted axon growth on RGMa substrate compared to controls, but unlike the latter, this axon outgrowth promoting activity is reduced and not enhanced by GGPP. The lactone prodrug form of lovastatin may thus have axon growth promoting activities beyond HMG-CoA reductase inhibition, likewise other statin class of compounds and their various derivatives.

How exactly does a reduction in axonal or growth cone cholesterol *per se* promote axon outgrowth? Given the fundamental importance of cholesterol to plasma membrane structure and function, the answer to this question may not be intuitively obvious, but the following may be plausible (**Figure 2**). Cholesterol content is a determinant of membrane fluidity, and it is possible that alterations in growth cone plasma membrane fluidity will influence axon outgrowth. This point may be particularly relevant for the extension of injured axons in adult neurons, as it has been hypothesized that regeneration of these may not involve the typical actin-myosin molecular motors that guide embryonic growth cones extension in developing axons. Rather, non-growth cone-mediated axon elongation mechanisms may be at work in extending injured axons in the mature CNS (Rodemer et al., 2020), and these may be aided, albeit in yet unclear ways, by an increase in membrane fluidity.

Cholesterol is enriched in lipid rafts, which are important for plasma membrane protein trafficking and targeting. Disruption of lipid rafts at the growth cone affects particularly the plasma membrane targeting of Glycosyl-phosphatidylinositol (GPI)-anchored proteins. In this regard, it is notable that the Nogo-66 receptor family of proteins (NgR1-3) (Borrie et al., 2012), which transduces growth inhibitory signals from myelin-associated inhibitory proteins such as Nogo-A, Myelin-associated glycoprotein (Wong et al., 2002), oligodendrocyte-myelin glycoprotein (Wang et al., 2002) and CSPGs (Dickendesher et al., 2012), are GPI-anchored proteins. Furthermore, the NgR co-receptor, p75NTR, could also be localized at lipid rafts (Higuchi et al., 2003). With cholesterol depletion leading to lipid raft disruption, a reduced targeting of NgRs to the growth cone surface would conceivably enhance axon outgrowth. NgRs are indeed segregated into lipid rafts in rat brain and Nogo-66 signaling has been shown to be inhibited by cholesterol depletion (Yu et al., 2004). In the same vein of thought, neogenin is also recruited to and interacts with the axonal growth inhibitor RGMa at lipid rafts, and the disruption of this interaction promoted axon outgrowth (Tassew et al., 2014).

It would also be interesting to see if cholesterol depleting or synthesis inhibition impairs targeting or expression of other receptors for axon outgrowth inhibitors. These would include the other Nogo receptors paired immunoglobulin-like receptor B (Atwal et al., 2008), and sphingosine 1-phosphate receptor 2 (Kempf et al., 2014), as well as the CSPG receptors protein tyrosine phosphatase σ (Shen et al., 2009) and leukocyte common antigen-related phosphatase (Fisher et al., 2011). Paired immunoglobulin-like receptor B in particular has been shown to act in terms of axon outgrowth inhibition in association with $p75^{NTR}$ (Fujita et al., 2011), while members of the protein tyrosine phosphatase family are known to be localized to lipid rafts (Caselli et al., 2002). Furthermore, growth cone repulsive molecules such as Semaphorin 7A and Ephrin-A are also GPI-anchored proteins (Um and Ko, 2017). These repulsive axon guidance molecules tend to be upregulated after injury and could contribute to inhibition of axon regeneration (Coulthard et al., 2012; Kopp et al., 2010). Cholesterol depletion and disruption of lipid rafts may also diminish their axon regeneration inhibitory effect.

Lipid rafts are also signaling hubs for a number of growth signaling receptors (Mollinedo and Gajate, 2020). In this context, it is notable that Nystatin treatment of hippocampal neurons seems to promote Akt phosphorylation and increase nitric oxide levels (Roselló-Busquets et al., 2020). The basis for these events are at present unclear, although these may be a consequence of perturbed growth receptor signaling processes that are otherwise more regulated or confined. These lipid raft disruption-enhanced signaling events may result in expression of axon outgrowth promoting factors such as growth-associated protein 43, and could also explain why cholesterol depletion promoted not just axon outgrowth, but also neuronal survival (Shabanzadeh et al., 2021), another key feature for successful regeneration. Neogenin's recruitment to lipid raft is dependent on bone morphogenetic protein (BMP)-

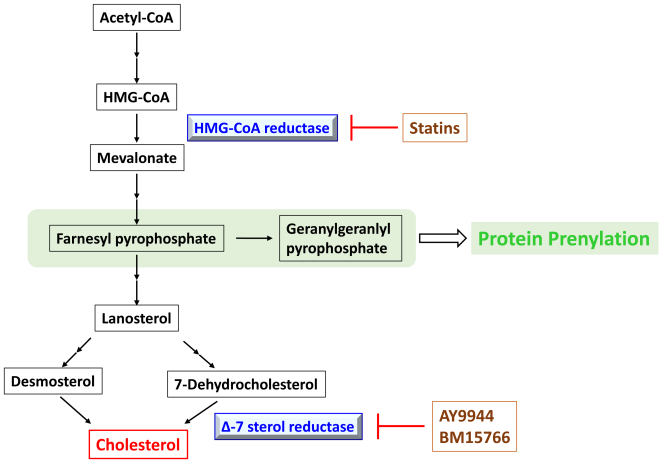


**Figure 1 | A highly simplified diagram of the cholesterol synthesis pathway, showing key intermediates and enzymes described in text.** Consecutive arrows indicate undefined number of steps involving metabolites not shown. The rate-limiting step of this pathway is catalyzed by HMG-CoA reductase, which could be inhibited by statins. Statins will thus block the production of not only cholesterol, but also mevalonate-derived farnesyl pyrophosphate and genarylgenaryl pyrophosphate that are substrate for protein prenylation. The final step of conversion of 7-Dehydrocholesterol to cholesterol is catalysed by Δ7-sterol reductase. Ay9984 and BM15766 are inhibitors of this enzyme. See text for more details. HMG-CoA: 3-Hydroxy-3-methylglutaryl-CoA.

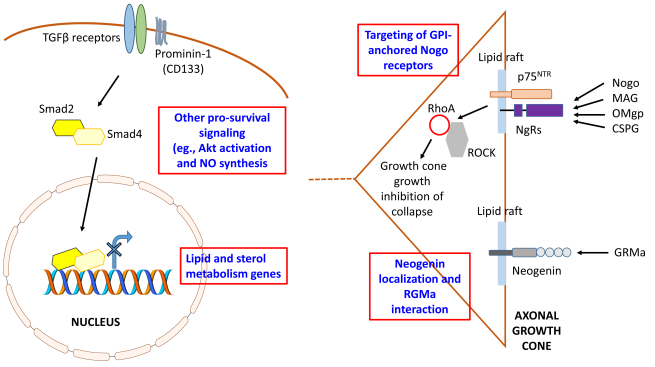


**Figure 2 | A schematic diagram depicting some of the possible cellular events and mechanisms underlying enhanced axon regeneration resulting from either cholesterol depletion or cholesterol synthesis inhibition.** Cholesterol depletion or cholesterol synthesis inhibition disrupts axonal or growth cone lipid rafts, which affects targeting and localization of NgRs and the co-receptor $p75^{NTR}$, transducers of axon growth inhibitory signals from myelin-associated inhibitors Nogo, MAG and OMgp and the injury-elevated CSPGs, as well as Neogenin, which interaction with GRMa exerts axon growth inhibition. Cholesterol depletion or cholesterol synthesis inhibition also depletes isoprenoids and inhibits prenylation of RhoA, which together with its effector ROCK mediates growth cone growth inhibition or collapse. The stem cell marker Prom1 is important for the promotion of axon inhibition through downregulation of cholesterol synthesis via its interaction with transforming growth factor-β type 1 receptor and downstream Smad signaling. Cholesterol depletion may also promote neuronal survival through signalling events such as Akt phosphorylation and NO synthesis, which could aid regeneration. See text for more details. CSPG: Chondroitin sulfate proteoglycan; GPI: glycosylphosphatidylinositol; MAG: myelin-associated glycoprotein; NgRs: Nogo-66 receptors; OMgp: oligodendrocyte myelin glycoprotein; $p75^{NTR}$: p75 neurotrophin receptor; RGMa: repulsive guidance molecule-a; ROCK: Rho-associated protein kinase; Smad: small mothers against decapentaplegic; TGFβ: transforming growth factor β.

mediated signaling that could be antagonised by noggin (Tassew et al., 2014). BMPs are members of the transforming growth factor-β superfamily, and canonical signaling from BMP and their receptors occurs via Smads (Orlova et al., 2016). This appears to be somewhat connected with the finding on Prom1-regulated Smad signaling which resulted in reduction in cholesterol synthesis and axon outgrowth (Lee et al., 2020). Further work would be required to properly resolve and clarify these connections, as well as the basis of enhanced pro-survival signaling resulting from cholesterol depletion.

## Implications and Caveats

The recent findings discussed above suggest that cholesterol depletion or synthesis inhibition promotes axon regeneration, at least for neurons *in vitro* and in rodent models. The findings also implied that cholesterol depletion or inhibition of its synthesis is exploitable in therapeutic approaches for the enhancement of axon (particularly those of CNS neurons) regeneration after injury, either by pharmacological or genetic manipulations. It is conceivable that either systemic or localized/carrier-targeted delivery of cholesterol depleting compounds, or a combination thereof, could be beneficial for axon regeneration in peripheral or CNS nerve lesions. This would also be somewhat in line with the notion that dysregulated cholesterol homeostasis contributes to several neurodegenerative diseases (Dai et al., 2021), such as Alzheimer's disease (Sáiz-Vazquez et al., 2020; Samant and Gupta, 2020), Parkinson's disease (García-Sanz et al., 2020) and Huntington's disease (González-Guevara et al., 2020). Cholesterol lowering drugs such as statins do have demonstrated benefits in some of the neurodegenerative disease models in animals (Saeedi Saravi et al., 2017; Langness et al., 2021).

However, several caveats should be heeded for the notions above. One of these would pertain to the effects any treatment might have on the different cell types at the injury/regenerating site. While specific cholesterol depletion from axonal growth cones may enhance axon outgrowth, its depletion from oligodendrocytes may impair myelination. One also needs to be mindful of cholesterol's essential structural and biochemical functions, and remember that targeted disruption of the gene encoding HMG-CoA reductase resulted in early embryonic lethality in mice (Ohashi et al., 2003). It is conceivable that a drastic or severe reduction of cholesterol would have systemically or neuronal specific detrimental effects. A threshold amount of cholesterol would likely be important for the proper functioning of key signaling molecules that modulates neuronal function and survival.

Beyond the obvious need for sufficient cholesterol to avoid hypomyelination, two other examples below portray a need for the maintenance of sufficient cholesterol in neurons. Firstly, cholesterol is known to modulate synaptic transmission (Krivoi and Petrov, 2019; Korinek et al., 2020) and changes in cholesterol levels could thus affect synaptic strength and plasticity (Wang and Zheng, 2015). Secondly, the activity and signaling of Sonic hedgehog (Chen et al., 2018), which is important for neuronal survival, neurogenesis and neural regeneration (Yamada et al., 2018; Dobbs et al., 2019), is critically dependent on its covalent modification by cholesterol (Riobo, 2012). Any therapeutic manipulation of cholesterol levels to promote axon regeneration would need to be carefully weighted and precisely executed. Much more therefore remains to be learned before this approach can become clinically translatable.

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