Axon regeneration: membrane expansion and lipidomics

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Axon regeneration requires protein synthesis and membrane expansion. The presence of granular material was discovered in the perikaryon of neurons but not axons using Nissl staining (Gomes, 2019). This is one of the characteristic features of neurons and we now know that RNA content of the granular material stains with Nissl stain. Although no Nissl granules are present in axons, mRNA can be shuttled to distal axons for local protein synthesis. Axonal protein synthesis only makes up a small portion as the majority occurs in the perikaryon. The required membrane expansion and protein synthesis machinery are still present in the adult central nervous system (CNS) axon. It is conceivable that regeneration of severed or dysfunctional axons, such as those present after traumatic optic nerve or spinal cord injury and due to elevated intraocular pressure in glaucoma, occurs with possible membrane sealing, growth cone formation, and membrane expansion (Meehan et al., 2021). Axon sealing after injury as well as growth are likely to be mediated by membrane expansion (Pfenninger et al., 2003). The majority of membrane biogenesis and *de novo* lipid synthesis occurs in the neuron's perikaryon. Vesicles are formed through the native secretory pathway and subsequently transported anterogradely to the distal axon for insertion. Vesicles can be seen accumulating at the growth cone's transitional zone, likely waiting for a regulatory signal to begin membrane fusion.

Although lipid biogenesis occurs in the perikaryon/soma, lipid remodeling and redistribution is present and necessary in the distal axon. Using *in vitro* compartmental studies, it has been shown that the furthest axons may even synthesize their own phospholipids with the presence of appropriate precursors (for example, Choline). This is not surprising as vesicle transport becomes less efficient as the axon distance increases. Local synthesis, remodeling, and redistribution allows for acute responses to external and internal stimuli. Responses are essential as the axon navigates surrounding tissues, using guidance cues, towards its end target. When a growth cone interacts with an inhibitory guidance cue, an unequal membrane expansion must occur biased towards the same side as the guidance cue. This ultimately leads to the axon turning away. Local axon lipid remodeling and transport can occur readily between bridged organelles, such as smooth endoplasmic reticulum (ER), mitochondria, and plasma membrane. Intimate connections between the ER and plasma membrane and the ER and mitochondria have been established in axons. Phosphatidylcholine production by axonal ER has been shown to be required for axon growth. At the plasma membrane, arachidonic acid produced by phospholipase A controls activity levels of membrane fusion and therefore, membrane expansion. Mitochondrial lipid metabolism and its effects on membrane expansion have not been explored. In terms of lipid redistribution, at the plasma membrane during membrane fusion, flippase and floppases must actively redistribute intrinsically curved lipids to adapt to membrane curvature shifts during membrane fusion. It appears the perikaryon provides the lipid foundation and building blocks while the distal axon creates the unique lipidomic shifts.

As lipids facilitate membrane expansion, could lipidomic studies of axon regeneration provide insights into lipids involved in neuronal membrane expansion? Membrane expansion and lipid biogenesis insight is likely best obtained through evaluation of several CNS neuron regenerative models (optic nerve, spinal cord), rather than single or limited model studies (**Figure 1**). Focusing on a single regenerative factor may create intrinsic biases based on a factor's specific signaling pathways. To understand axon regeneration as an overall process, it is better to evaluate many regenerative factors while controlling measurements and standardizing parameters, such as the length and volume of regrown axon, time post crush, transgenic nature of the animal, and the injury method used. We have discovered the importance of these lipidomic parameters as identifying significant lipid species can be difficult.

Not all regenerative factors are equal and should not be arbitrarily chosen for lipidomic analysis. A common fault for some regenerative factors is axon misguidance. This misguidance is often displayed as an axon with excessive turning, u-turning, and an overall convoluted pathway. The explanation for this is still being studied but is partially due to the inhibitory signals and neuroglia found in lesion sites. This misguidance can even lead to axons never traversing the lesion site. Although the axon is considered "regenerating" it will not lead to reinnervation in this misguided state. Using any regenerated axon data would lead to higher prioritization of intrinsically curved lipids as the axon would have extensive curvature. As such, regenerative factors should be chosen based on the extent to which they induce long distance regeneration (away from the lesion site). During development, retinal ganglion cell axons can reach their targets in a matter of days. However, in the adult mammalian system, it can take upwards of 6 weeks to reach complete axon regeneration. Earlier time points, such as 3 days post injury, will not provide significant lipidomics involved in regeneration as the axons have not traversed the lesion site. Concurrently, the induced lipid changes may still be an injury response at earlier time points compared to the lipids involved in axon growth at later time points.

It is important to note that long-distance regeneration in mammalian peripheral nervous system (PNS) with substantial functional recovery occur in adults with

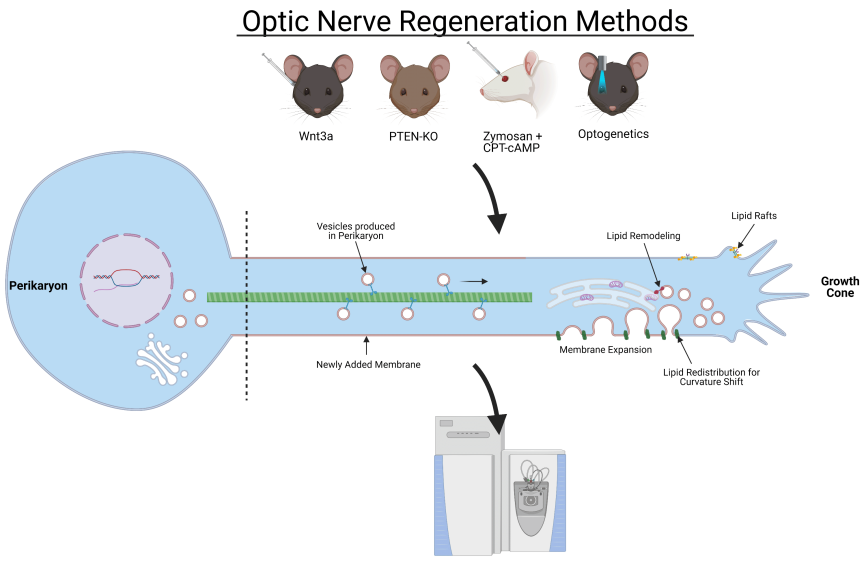

Figure 1 | **Comparative lipidomics of optic nerve regeneration models.**
Lipidomic comparison studies between several optic nerve regeneration models may provide insight into prioritized lipid pathways involved in processes such as membrane expansion, lipid remodeling. There are currently four published lipid profiles for optic nerve regeneration: Wnt3a-induced, Zymosan-induced, PTEN-knockout (KO), and optogenetics. Comparative axonal lipidomics can be performed by isolating axons (dotted line).

ease. PNS regeneration with respect to adult regeneration show vast differences with CNS (Curcio and Bradke, 2018). One of the big differences of PNS regeneration is involvement of different cell types compared to CNS (Curcio and Bradke, 2018). A very limited lipidomics analyses such cell types in different PNS tissues have been conducted (Meyer Zu Reckendorf et al., 2020). Greater heterogeneity of PNS tissues also raise greater permutation of such comparisons and incorporate higher complexity in analyses. Nevertheless, a comparison of lipidome of PNS with CNS may also help identify lipids/metabolites that may be helpful to understand not only the differences in both regenerative abilities but may add to our increasing arsenal of molecules for promotion of CNS regeneration.

Our recent lipidomic study (Arcuri et al., 2021) is one such profile of regenerating neurons among a few others (Trzeciecka et al., 2019; Arcuri et al., 2020, 2021). Several recent investigations into members of different lipid classes and their critical role in neuron regeneration have underscored the importance of lipids towards regeneration of optic nerve and spinal cord neurons (Tassew et al., 2014, 2017; Yang et al., 2020; Shabanzadeh et al., 2021). For example, if we consider the physical structure of the membrane, phospholipids make up > 50% of the axonal membrane. In retinal ganglion cells, shifting triglyceride synthesis (energy storage) to phospholipid metabolism (membrane building) promotes axon regeneration in retinal ganglion cells. Specifically, Pcyt1a and Pcyt2, two lipid metabolic enzymes involved in production of phosphatidylcholine and phosphatidylethanolamine, respectively, were found to support axon regeneration. From a functional standpoint, receptors can be localized to lipid rafts in the growth cone. These receptors can be regeneration-promoting and -inhibiting depending on their response to cellular signals. Disruption of lipid rafts and regeneration-inhibiting receptor association to lipid rafts through cholesterol reduction was shown to promote axon growth in retinal ganglion cells and functional recovery after spinal cord injury. On the other hand, the presence of lipid rafts is essential for exosomes containing regeneration-promoting factors to induce axon growth. Although this dichotomy exists, the indispensability of lipid rafts should be further elucidated through evaluation of regenerative factors that induce successful long-distance axon regeneration and reinnervation only. It should be emphasized that lipidomics of these regenerative factors should be evaluated using *in vivo* models. Neuroglia can contribute to a growing axon's lipid profile. For example, extracellular lipoproteins from macrophages are capable of shuttling cholesterol to a growing axon's tip. The *in vivo* lipidomic studies may have contributions from neuron and neuroglia. *In vitro* studies are usually limited to short distance regeneration and do not fully capture issues encountered *in vivo*. Fractionation to decipher lipidome contributed by neuron and neuroglia will help understand their respective contribution towards long-distance neuron regeneration. If the long-distance regenerative lipidomics is partitioned further, then the combination of growth cone lipidome (Chauhan et al., 2020) and, axonal lipidome during expansion may provide us a good deal of insight about regenerating axons, functionally and structurally.

The lipidomics of neuronal tissue has several limitations. Such tissues are, in reality, mixed tissues, with significant heterogeneity. The pan-lipidomic methods may be differentially distinct for extraction and analysis of lipids of different classes. Purified axon, neuron and fractionated tissue/cells may be necessary for more complete and deep profiling as well as better understanding. The fractionation methods itself are problematic as there is no perfect method and almost all methods are really enrichment. Not to mention that lipid exchange between membranes and organelles occurs readily. Also, the isolated cells may be additive and may even enable cleaner analysis of secretome but they are not same as *in situ* secretome or *in situ* cells. Recent advances in imaging mass spectrometry along with kinetic histochemistry may enable lipid localization and complement the deep profiling data with combined approaches. Our lipidomic analysis from limited models of optic nerve regeneration already points out differences reflective of heterogeneity, extraction and other aspects, as noted above. Aspects of tissue lipid distribution can be evaluated using imaging mass spectrometry, which utilizes fixed tissue sections and matrix-assisted laser desorption/Ionization to create a lipid intensity image. This technique has already been used to evaluate the lesion site after optic nerve crush and after traumatic brain injury. Lipidomic analyses can help complement at another level. Lipidomics integrated with other OMICS (transcriptomics, proteomics, and metabolomics) can help build confident prioritization of pathways that may be critical for adult neuronal regeneration (Chauhan et al., 2020). Combinatory strategies using these pathways and already established regenerative factors could be used to improve therapeutic potential as well.

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