

● PERSPECTIVE

The role of muscle LIM protein in the nervous system

Unlike the peripheral nervous system (PNS), the central nervous system (CNS) has a low intrinsic regenerative capacity and has mechanisms that actively suppress axon regrowth, for example, glial scarring and myelin inhibition (Fischer, 2012). Even in the PNS, which has the principle ability to regenerate injured axons, functional recovery remains limited, particularly in cases where the nerve target has become unresponsive to re-innervation over time due to an insufficient axonal growth rate (Diekmann and Fischer, 2015). Progress towards robust neuroregenerative therapies depends upon an understanding of the relevant signaling and cytoskeletal proteins that drive and control axon extension. Muscle LIM protein (MLP), also known as cysteine and glycine-rich protein 3, was recently discovered to be one such protein that is expressed in regenerating rat neurons and whose overexpression can promote the axon regeneration of adult central, and peripheral neurons of different species (Levin et al., 2019).

As its name suggests, MLP is assumed to be a muscle-specific member of the LIM-domain protein superfamily. It is a cysteine-rich protein that can be detected in the early stage of terminal muscle cell differentiation (Arber and Caroni, 1996). Many functions have been reported to be associated with myocyte cytoarchitecture through cross-linking actin into bundles, or its accumulation in the nucleus in response to mechanical stress (Paudyal et al., 2016). Mutations in MLP, or its ectopic expression, are associated with a range of muscle-related diseases (such as hypertrophic cardiomyopathies) and various skeletal myopathies, respectively (Vafiadaki et al., 2015).

The first hints as to MLP's expression in the CNS and its regenerative role there came from a microarray study, which had characterized the gene expression patterns of axotomized retinal ganglion cells (RGCs) (Fischer et al., 2004). The combination with an inflammatory stimulation, which transforms adult RGCs into a regenerative state due to a glial release of interleukin-6-like cytokines, further upregulates *Mlp* expression. A decade later, Levin et al. (2014) also found the expression of MLP in the uninjured nervous system. Meanwhile, not only the mRNA but also the protein itself was identified to be transiently expressed in retinal cholinergic amacrine cells during the first few weeks after birth (Levin et al., 2014). MLP's function in these neurons during this postnatal period, however, remains to be elucidated.

Later, experiments were performed in the PNS showing that specific sensory neurons responded to nerve injury by the induction of MLP expression (Levin et al., 2017). The study found MLP in the lumbar dorsal root ganglia (DRG) sensory neurons in response to a sciatic nerve injury in rats. Interestingly, MLP expression was restricted to a small subpopulation of DRG neurons that did not appear to co-stain with any established subtype markers. This finding included immunostaining the heavy-chain neurofilament to label large-diameter myelinated neurons, calcitonin gene-related peptide/substance P to mark peptidergic nociceptors, and finally, isolectin B4 (IB4) itself to stain non-peptidergic nociceptive neurons. Surprisingly, of the MLP-positive neurons observed at 2 days after the nerve crush, almost half of them could be co-stained for IB4; however, by day 7, this number had fallen to just 1%. More-

over, the nerve injury was causing the non-peptidergic nociceptive sensory neurons to lose their canonical marker (IB4), hiding their identity. The first evidence of this came when culturing the cells *in vitro*, where 58% of neurons were positive 2 hours after plating, whereas after 5 days, only 30% were IB4-positive, while the other subtype markers' levels remained constant. Treatment with glial-derived neurotrophic factor delayed axotomy-induced downregulation of IB4 sensitivity without altering the total number of neurons, which then brought the number of co-labeled neurons up to 51% (from 11%). Thus, as the glial-derived neurotrophic factor receptor is another marker for non-peptidergic nociceptive sensory neurons, it confirms that the majority of MLP-positive neurons belong to this group in the PNS (Levin et al., 2017). As in amacrine cells, the function of endogenous MLP expression in this subpopulation of sensory neurons is not yet clear either. However, Levin et al. (2019) now showed that MLP overexpression in cultured DRG sensory neurons resulted in a doubling of axon growth and growth cone size, even though naive or damaged neurons of mice do not express MLP. Whether endogenously expressed MLP in rats also increases the regenerative capacity of IB4-positive sensory neurons is currently unknown.

Levin et al. (2019) also showed most recently MLP expression (also on the protein level) in injured and growth-stimulated RGCs of adult rats, allowing us to scrutinize its functional role in this context. Interestingly, inflammatory stimulation alone was not enough to drive MLP expression, yet its timing matched that of RGCs transdifferentiating into a regenerative state, beginning 2–3 days after injury. When MLP was exogenously overexpressed early using a viral vector, RGCs substantially increased axon growth compared to naturally induced MLP, which peaks later at 5–7 days after axotomy. MLP's role in these experiments was confirmed by suppression of the regenerative neurite growth after shRNA against MLP or the use of a dominant-negative version of the protein in growth-stimulated rat RGCs *in vivo*. Experiments in cell culture also showed a correlation with a regenerative state, with MLP-positive RGCs displaying stronger spontaneous neurite outgrowth after stimulation. Despite the positive effect on axon growth, neither the knockdown nor overexpression of MLP affected the survival of axotomized RGCs or neurite growth on inhibitory myelin (Levin et al., 2019).

When looking at the effect of MLP *in vitro*, primary cultures from rat RGCs showed a doubling of neurite outgrowth speed if the neurons had been transduced to express MLP before harvest. Remarkably, mice do not have any endogenous MLP in neurons, yet exogenous transduction of MLP still promoted axon regeneration, suggesting that it acts through a conserved mechanism (Levin et al., 2019). Functionally, MLP is known to respond to biomechanical stress in myocytes by accumulating in the nucleus and modulating gene expression (Paudyal et al., 2016). It is also present in the cytoplasm, axons, and growth cones of postnatal amacrine cells (Levin et al., 2014). Levin et al. (2019) therefore addressed whether either one or both of these localization targets (nucleus, cytoplasm, growth cone) are needed for the growth cone-promoting effect by transducing RGCs with MLP fused to a nuclear localization signal. This MLP-fusion-protein did not reach the growth cones and subsequently failed to enhance axon regeneration of RGCs in both culture and *in vivo* (Levin et al., 2019). Upon closer inspection of the growth cone, MLP seemed to exert its effect through interaction with filamentous actin. In myocytes, it was shown that the self-association of MLP promoted the bundling of actin filaments by

acting as a cross-linking agent (Hoffmann et al., 2014). The self-association behavior of MLP was demonstrated to function through its N-terminal LIM domain, whereas its actin filament-binding properties were conveyed through the C-terminal LIM domain (Hoffmann et al., 2014). Similarly, its actin bundling properties in the axonal growth cone are likely dependent on this cross-linking ability as treatment with the actin depolarization agent latrunculin A abolished MLP's neurite growth-promoting effects (Levin et al., 2019). More specifically, MLP is thought to increase filopodia formation, which is, in turn, a prerequisite for highly motile growth cones (Levin et al., 2019).

Coming full circle to what started it all, the microarray study that first identified *Mlp* as being upregulated in damaged neurons put it among other genes such as *Gap43* and *Sprr1a* (Fischer et al., 2004). Interestingly, both of the other two regeneration-associated genes are also involved in the regulation of actin dynamics in axons. MLP, on the other hand, seems to mainly enhance axon growth by facilitating filopodia formation through actin cross-linking, as opposed to promoting actin poly- or depolymerization processes. The different pathways used by these proteins are highlighted by an increase in the growth cone size being reported only in MLP overexpression (Levin et al., 2019). Also, *GAP43* expression depends on JAK/STAT3 signaling, whereas MLP requires mTOR activity (Leibinger et al., 2013; Levin et al., 2019).

Now that MLP has been shown to affect the CNS *via* recent RGC experiments, it begs the questions of whether combining it with other molecules known to have neuroprotective and disinhibitory effects can synergistically promote neuroregeneration in the visual system or whether neurons of the spinal cord or cortex respond similarly. AAV2 viral vectors are clinically accepted therapeutic tools in the human nervous system and can be used to specifically transduce neurons to confer neuroprotection for example by caspase-2 knockdown (Ahmed et al., 2011) along with MLP overexpression.

In the PNS, the role of endogenously induced MLP in axotomized non-peptidergic nociceptor sensory neurons awaits further characterization, despite the finding that exogenously expressed MLP promotes the axon growth of adult sensory neurons *in vitro* (Levin et al., 2019). Conditional PNS knockout and overexpression upon viral transduction would be useful for determining MLP's potential regenerative role *in vivo*, particularly in relation to the age-dependent decrease of axon regeneration capacity. Knockout experiments could also uncover whether MLP is involved in axon growth during embryonic development. Furthermore, it will also be interesting to see if humans endogenously express MLP in response to neural injury, or whether like in mice, it is absent yet still capable of promoting regeneration when exogenously transduced.

This work was supported by the German Research Foundation (FI 867/12, to DF).

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Received: February 23, 2019

Accepted: April 8, 2019

doi: 10.4103/1673-5374.259607

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Peer review: Externally peer reviewed.

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Open peer reviewer: Michele R. Colonna, University of Messina, Italy.

Additional file: Open peer review report 1.

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P-Reviewer: Colonna MR; *C-Editors:* Zhao M, Li JY; *T-Editor:* Jia Y