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Morpholino studies shed light on the signaling pathways regulating axon regeneration in lampreys

Daniel Sobrido-Cameán[†], Antón Barreiro-Iglesias[*]

Lampreys are one of the most ancient extant vertebrates and they have become an animal model of interest for the study of spontaneous axon regeneration after a traumatic central nervous system injury. Contrary to most mammals, lampreys recover locomotion after a complete spinal cord injury (SCI). During recovery from SCI, some of the descending axons in lampreys regenerate through the injury site and reinnervate caudal levels of the spinal cord. Interestingly, the brainstem of lampreys contains 36 giant descending neurons that can be identified individually and that show very different survival and regenerative abilities after a complete SCI (Jacobs et al., 1997; see Barreiro-Iglesias, 2015), even when their axons are found in similar locations in a spinal cord that is permissive for axonal regrowth. Some of these identifiable neurons are considered "good" regenerators (they regenerate their axon more than 55% of the times) and others are considered "bad" regenerators (they regenerate their axon less than 50% of the times) (**Figure 1**). This offers a model in which the intrinsic mechanisms regulating neuronal survival and axonal regrowth can be studied *in vivo* and at the level of individual neurons. First, one can use this model to find genes showing differential expression between "good" and "bad" regenerator neurons, and then try to perform functional studies by manipulating their expression or their action. As in any other animal model, drugs can be used for this purpose (Fogerson et al., 2016; Romaus-Sanjurjo et al., 2018; Sobrido-Cameán et al., 2019, 2020), but ideally genetic manipulations are also needed to confirm drug effects or to manipulate the expression of genes for which no drugs are available.

Unfortunately, lampreys, like the sea lamprey (*Petromyzon marinus*; which is the most commonly used lamprey species in SCI studies), have a very complex and long life cycle. Sea lampreys have a long filter-feeding larval stage in the river (5 to 7 years), a transformation and an adult parasitic stage in the sea before they return to the river to breed and die. This has precluded the generation of stable mutant or transgenic lampreys in the laboratory. Transient genetic knock down can be a good alternative to the use of permanent genetic manipulations for functional studies. So far, morpholinos (available from Gene Tools, LLC) are the only genetic tool that has been proven effective for SCI studies in mature larval lampreys (Zhang et al., 2015; Fogerson et al., 2016; Hu et al., 2016; Chen et al., 2017; Romaus-Sanjurjo et al., 2018; Sobrido-Cameán et al., 2019; Rodemer et al., 2020).

Morpholinos: Morpholinos are synthetic antisense oligonucleotides (around 20–25 nucleotides) that bind messenger RNAs (mRNAs). Morpholinos are different from natural nucleic acids, since they contain methylenemorpholine rings replacing the ribose or deoxyribose sugar moieties and non-ionic phosphorodiamidate linkages replacing the anionic phosphates. Each morpholine ring positions one of the standard DNA bases for pairing, so that a morpholino oligo specifically binds to its complementary target site. Morpholino binding blocks access of cell components to the mRNA target site. This allows to block mRNA translation (translation-blocking morpholinos), mRNA splicing (splice-blocking morpholinos), inhibit micro-RNA action (or their targets) or block ribozyme activity (Moulton, 2016).

Morpholino studies after SCI in lampreys: Conveniently, when morpholinos are applied at the site of injury after a complete spinal cord transection in lampreys they are taken up by the axotomized descending axon and are retrogradely transported to the neuronal soma of neurons located in the brainstem. This retrograde transport has been proven by labeling morpholinos with fluorescent molecules (Zhang et al., 2015; Fogerson et al., 2016; Hu et al., 2016; Chen et al., 2017; Sobrido-Cameán et al., 2019; Rodemer et al., 2020). Application of morpholinos after SCI in lampreys has been mainly done by soaking the morpholino solution in a small piece of Gelfoam (available from Pfizer) that is then placed at the injury site (Zhang et al., 2015; Fogerson et al., 2016; Hu et al., 2016; Romaus-Sanjurjo et al., 2018; Rodemer et al., 2020). In some studies, the Gelfoam was left at the site of injury for only a couple of hours and it was then removed before returning the animals to the aquaria (Chen et al., 2017). However, fluorescent morpholino labeling has also shown that a morpholino solution directly applied to the rostral stump of the transected spinal cord allows for the retrograde transport of the morpholino (Sobrido-Cameán et al., 2019).

As indicated in **Additional Table 1**, 7 studies have used translation- (4 studies) or splice-blocking (3 studies) morpholinos to knock down the expression of 7 target mRNAs/pre-mRNAs in descending neurons of lampreys after a complete SCI. In these studies, control animals with an SCI received either a standard control morpholino supplied by Gene Tools or a custom-made 5-base mismatch morpholino. 5-Base mismatch morpholinos contain the same sequence of the active morpholino with changes in 5 of the bases (therefore, they should not bind the target mRNA). To confirm that the morpholinos knocked down the expression of the target mRNAs in brainstem neurons these different studies used Western Blot, *in situ* hybridization and/or immunofluorescence techniques to reveal decreased expression of the target mRNA/protein (**Additional Table 1**).

The first study reporting the use of morpholinos in the lamprey model of SCI was published in 2015 by Zhang et al. These authors used translation-blocking morpholinos directed against the sea lamprey neurofilament subunit NF180 and showed that inhibition of neurofilament expression inhibits axon regeneration after SCI. Interestingly, NF180 morpholino application had no effect on the axon retraction that occurs initially after a complete SCI (**Figure 1**). This study provided functional data confirming that axonal regeneration in lampreys (and perhaps in the other vertebrates) probably depends on an internal protrusive force generated by the transport or assembly of neurofilaments in the distal axon instead of the canonical actin-dependent pulling mechanisms (Zhang et al., 2015).

Fogerson et al. (2016) used a translation blocking morpholino against the sea lamprey gamma-synuclein to show that selective accumulation and aggregation of synuclein leads to neurodegeneration

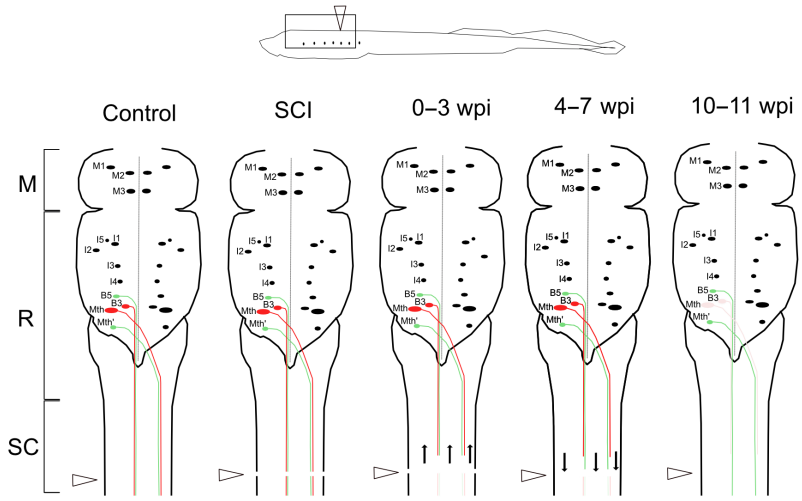

Figure 1 | **Schematic drawing of lateral view of a larval sea lamprey (at the top) and schematic drawings of the larval brainstem and spinal cord from a dorsal view (at the bottom).**
The location of some of the identifiable descending neurons (for most neurons only the soma is represented) in non-injured animals (control) and at different wpi: The axons of the "good" regenerator neurons B5 and Mth' (in green) and of the "bad" regenerator neurons Mth and B3 (in red) are shown as examples. Control: in control animals descending axons project to the spinal cord; SCI: the axons are transected at the site of injury (indicated by the arrowheads); 0–3 wpi: in the first wpi the injured axons mainly retract from the site of injury; 4–7 wpi: between 4 and 7 weeks after injury, the axons of good regenerator neurons re-grow through the injury site; 10–11 wpi: 10–11 weeks after the injury the axons of good regenerators have reinnervated the caudal spinal cord, while the bad regenerators suffer a delayed death by apoptosis. The box represents the position of the brain/spinal cord schematics. M: Mesencephalon; R: rhombencephalon; SC: spinal cord; SCI: spinal cord injury; wpi: weeks post-injury.

in bad survivor descending neurons after SCI. Synuclein morpholino knock down not only improved neuronal survival, but also increased the number of axons in the spinal cord after the SCI (Fogerson et al., 2016). Morpholino results were confirmed with the inhibitor of amyloidogenic protein aggregation CLR01, which also improved the survival of descending neurons in the brainstem (Fogerson et al., 2016). This study was the first to demonstrate, in any vertebrate model, that synuclein accumulation causes neurodegeneration after SCI as had been previously shown for neurodegenerative diseases like Parkinson's disease. Interestingly, recent work has shown that lentivirus downregulation of alpha-synuclein promotes functional recovery in rats after SCI (Zheng et al., 2019).

Application of translation-blocking morpholinos directed against the sea lamprey RhoA mRNA after a complete SCI caused a reduction in caspase activation in descending neurons, inhibited axon retraction in the rostral stump and increased axon regeneration through the injury site (Hu et al., 2017). Previous work in mammalian models of SCI had already shown the positive effects of RhoA inhibition after SCI. However, with results previously obtained in rodent models it was not clear whether the positive effects of RhoA inhibition were due to the promotion of true axon regeneration as opposed to collateral sprouting by spared axons. This study in the lamprey model shows that RhoA inhibition can enhance true axon regeneration and prevent retrograde apoptotic death after SCI (Hu et al., 2017).

Morpholino work has also allowed deciphering the role of the axon guidance receptor Neogenin during axonal regeneration (Chen et al., 2017, Chen and Shifman, 2019). Neogenin serves as a receptor for the repulsive guidance molecule. *In situ* hybridization data showed that this receptor is preferentially expressed in bad regenerators of the sea lamprey brainstem (Chen et al., 2017). Concordantly, the application of a Neogenin splice-blocking morpholino at the time of spinal cord transection promoted the regeneration of identifiable descending neurons with low or intermediate regenerative capacity (Chen et al., 2017). The incomplete regeneration induced by the Neogenin morpholino could be explained by the expression of multiple axonal guidance receptors in descending neurons, including UNC5 or Plexins (Barreiro-Iglesias et al., 2012, Chen et al., 2017). Further work by the same group showed that application of the same morpholino promotes neuronal survival in descending neurons by inhibiting caspase activation (Chen and Shifman, 2019). Importantly, increased neuronal survival and axonal regeneration after Neogenin morpholino application lead to improved behavioral recovery after a complete SCI in lampreys (Chen and Shifman, 2019).

Recent work from our group using morpholinos has also revealed the role of classical neurotransmitters in the regulation of axonal regrowth and neuronal survival after a complete SCI in lampreys (Romaus-Sanjurjo et al., 2018; Sobrido-Cameán et al., 2019). These studies showed opposing roles for serotonin and GABA during axonal regeneration. The use of translation-blocking morpholinos showed that endogenous serotonin inhibits axonal regeneration by activating 1A serotonin receptors present in brainstem descending neurons (Sobrido-Cameán et al. 2019). Activation of serotonin 1A receptors leads to a reduction in cyclic-AMP levels, which in turn could inhibit axonal regrowth (Sobrido-Caméan et al., 2019). In contrast, endogenous GABA acting through $GABA_B$ receptors expressed in identifiable descending neurons promotes both neuronal survival and axon regeneration in these neurons after SCI (Romaus-Sanjurjo et al., 2018). Interestingly, baclofen (a $GABA_B$ agonist used as an anti-spasticity medication in SCI patients) could be a drug of interest to promote neuroprotection and recovery after SCI in mammals, including humans (see de Sousa et al., 2021).

Finally, a recent study has reported surprising results in which morpholino knock down of the chondroitin sulphate proteoglycans receptor PTPsigma in sea lampreys impaired axon regeneration and neuronal survival (Rodemer et al., 2020). These results were unexpected since expression data revealed that PTPsigma was predominantly expressed in bad regenerators after SCI in lampreys and chondroitin sulphate proteoglycans are known to inhibit axonal sprouting/regrowth after SCI in mammals. Future work should try to decipher the molecular mechanisms by which PTPsigma knock down leads to suppression of axon regeneration and neuronal survival in lampreys. One possibility already suggested by Rodemer and colleagues (2020) is that PTPsigma could be suppressing the activation of autophagy pathways, which could drive cell death and impair axon regeneration following morpholino knockdown.

Conclusions: All these studies from the last 6 years show that morpholino work in the lamprey model of SCI can provide interesting new insights on the role of different molecules and signaling pathways in axon regeneration and neuronal survival in vertebrates. This is also providing new molecular targets to promote recovery after SCI in mammalian models.

Recent transcriptomic data has provided new possible mRNA targets to be manipulated with morpholinos in the lamprey model of SCI (e.g., different kinases or KLF transcription factors; Herman et al., 2018; Sobrido-Cameán et al., 2020). So, future morpholino work could focus on these new genes. New studies could also attempt to target several mRNAs simultaneously to determine if combinatorial approaches can improve the results obtained with single morpholinos. Future methodological work should also attempt to develop new genetic tools to complement morpholino work in the lamprey model of SCI. Application of small interfering RNAs or CRISPR/Cas tools to giant descending neurons of lampreys could be an interesting way to confirm and complement morpholino work. This would provide robustness to morpholino studies in lampreys to control for possible off-target effects of morpholinos.

Daniel Sobrido-Cameán[†], Antón Barreiro-Iglesias[*]

Department of Functional Biology, Faculty of Biology, CIBUS, Universidade de Santiago de Compostela, Santiago de Compostela, A Coruña, Spain (Sobrido-Cameán D, Barreiro-Iglesias A)

†Current address: Department of Zoology, University of Cambridge, Cambridge, UK

***Correspondence to:** Antón Barreiro-Iglesias, PhD, anton.barreiro@usc.es.

https://orcid.org/0000-0001-8239-2965 (Daniel Sobrido-Cameán);

orcid.org/0000-0002-7507-080X (Antón Barreiro-Iglesias)

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Additional file:

Additional Table 1: *Morpholino studies in the lamprey model of spinal cord injury.*

References

Barreiro-Iglesias A (2015) "Bad regenerators" die after spinal cord injury: insights from lampreys. Neural Regen Res 10:25-27.

Barreiro-Iglesias, A, Laramore, C, Shifman, M I (2012) The sea lamprey UNC5 receptors: cDNA cloning, phylogenetic analysis and expression in reticulospinal neurons at larval and adult stages of development. J Comp Neurol 520:4141-4156.

Chen J, Laramore C, Shifman MI (2017) The expression of chemorepulsive guidance receptors and the regenerative abilities of spinal-projecting neurons after spinal cord injury. Neuroscience 341:95-111.

Chen J, Shifman MI (2019) Inhibition of neogenin promotes neuronal survival and improved behavior recovery after spinal cord injury. Neuroscience 408:430-447.

Fogerson SM, van Brummen AJ, Busch DJ, Allen SR, Roychaudhuri R, Banks SM, Klärner FG, Schrader T, Bitan G, Morgan JR (2016) Reducing synuclein accumulation improves neuronal survival after spinal cord injury. Exp Neurol 278:105-115.

Herman PE, Papatheodorou A, Bryant SA, Waterbury CKM, Herdy JR, Arcese AA, Buxbaum JD, Smith JJ, Morgan JR, Bloom O (2018) Highly conserved molecular pathways, including Wnt signaling, promote functional recovery from spinal cord injury in lampreys. Sci Rep 8:742.

Hu J, Zhang G, Rodemer W, Jin LQ, Shifman M, Selzer ME (2017) The role of RhoA in retrograde neuronal death and axon regeneration after spinal cord injury. Neurobiol Dis 98:25-35.

Jacobs AJ, Swain GP, Snedeker JA, Pijak DS, Gladstone LJ, Selzer ME (1997) Recovery of neurofilament expression selectively in regenerating reticulospinal neurons. J Neurosci 17:5206-5220.

Moulton JD (2016) Guide for morpholino users: toward therapeutics. J Drug Discov Dev Deliv 3:1023.

Rodemer W, Zhang G, Sinitsa I, Hu J, Jin LQ, Li S, Selzer ME (2020) PTPσ knockdown in lampreys impairs reticulospinal axon regeneration and neuronal survival after spinal cord injury. Front Cell Neurosci 14:61.

Romaus-Sanjurjo D, Ledo-García R, Fernández-López B, Hanslik K, Morgan JR, Barreiro-Iglesias A, Rodicio MC (2018) GABA promotes survival and axonal regeneration in identifiable descending neurons after spinal cord injury in larval lampreys. Cell Death Dis 9:663.

Sobrido-Cameán D, Robledo D, Romaus-Sanjurjo D, Pérez-Cedrón V, Sánchez L, Rodicio MC, Barreiro-Iglesias A (2020) Inhibition of gamma-secretase promotes axon regeneration after a complete spinal cord injury. Front Cell Dev Biol 8:173.

Sobrido-Cameán D, Robledo D, Sánchez L, Rodicio MC, Barreiro-Iglesias A (2019) Serotonin inhibits axonal regeneration of identifiable descending neurons after a complete spinal cord injury in lampreys. Dis Model Mech doi: 10.1242/dmm.037085.

Zeng H, Liu N, Yang YY, Xing HY, Liu XX, Li F, La GY, Huang MJ, Zhou MW (2019) Lentivirus-mediated downregulation of α-synuclein reduces neuroinflammation and promotes functional recovery in rats with spinal cord injury. J Neuroinflammation 16:283.

Zhang G, Jin LQ, Hu J, Rodemer W, Selzer ME (2015) Antisense morpholino oligonucleotides reduce neurofilament synthesis and inhibit axon regeneration in lamprey reticulospinal neurons. PLoS One 10:e0137670.

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Additional Table 1 Morpholino studies in the lamprey model of spinal cord injury

Target mRNA	Type of morpholino	Effect	Controls	References
Neurofilament NF180	Translation-blocking	Inhibited axonal regeneration	Standard control morpholino; Western blotting and NF180 immunofluorescence	Zhang et al., 2015
Gamma-synuclein	Translation-blocking	Increased neuronal survival (Nissl-staining) and increased axon plasticity (i.e., number of spinal cord axons)	5-Base mismatch morpholino; synuclein immunofluorescence	Fogerson et al., 2016
Neogenin	Splice-blocking	Increased axon regeneration	5-Base mismatch morpholino; *in situ* hybridization for Neogenin mRNA, Western blotting and TUNEL staining	Chen et al., 2017
RhoA	Translation-blocking	Increased neuronal survival (FLICA assay), inhibited axon retraction and increased axon regeneration	Standard control morpholino; Western blotting and RhoA immunofluorescence	Hu et al., 2017
GABAB1 subunit of the GABAB receptor	Splice-blocking	Inhibited axon regeneration	5-Base mismatch morpholino; *in situ* hybridization for gabab1 mRNA	Romaus-Sanjurjo et al., 2018
5-HT1A receptor	Translation-blocking	Increased axon regeneration	Standard control morpholino; 5-HT1A receptor immunofluorescence	Sobrido-Cameán et al., 2019
PTPsigma	Splice-blocking	Inhibited axon regeneration and reduced long-term neuronal survival	Standard Control morpholino; *in situ* hybridization for PTPsigma mRNA and Western blotting	Rodemer et al., 2020

FLICA: Fluorochrome-labelled inhibitors of caspases; 5-HT1A: serotonin receptor 1A.