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Reducing synuclein accumulation improves neuronal survival after spinal cord injury

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ABSTRACT

Spinal cord injury causes neuronal death, limiting subsequent regeneration and recovery. Thus, there is a need to develop strategies for improving neuronal survival after injury. Relative to our understanding of axon regeneration, comparatively little is known about the mechanisms that promote the survival of damaged neurons. To address this, we took advantage of lamprey giant reticulospinal neurons whose large size permits detailed examination of post-injury molecular responses at the level of individual, identified cells. We report here that spinal cord injury caused a select subset of giant reticulospinal neurons to accumulate synuclein, a synaptic vesicle-associated protein best known for its atypical aggregation and causal role in neurodegeneration in Parkinson's and other diseases. Post-injury synuclein accumulation took the form of punctate aggregates throughout the somata and occurred selectively in dying neurons, but not in those that survived. In contrast, another synaptic vesicle protein, synaptotagmin, did not accumulate in response to injury. We further show that the post-injury synuclein accumulation was greatly attenuated after single dose application of either the "molecular tweezer" inhibitor, CLR01, or a translation-blocking synuclein morpholino. Consequently, reduction of synuclein accumulation not only improved neuronal survival, but also increased the number of axons in the spinal cord proximal and distal to the lesion. This study is the first to reveal that reducing synuclein accumulation is a novel strategy for improving neuronal survival after spinal cord injury.

1. Introduction

Spinal cord injury (SCI) damages neurons, leading to widespread neurodegeneration and neuronal death (Hains et al., 2003; Viscomi and Molinari, 2014). In mammals, loss of neurons, along with poor capacity for regeneration, contributes to the permanent impairment of movement and sensation after SCI (Blesch and Tuszynski, 2009). Though injury-induced neurodegeneration limits the extent of regeneration and spinal cord repair, very little is known about the mechanisms that cause injury-induced neurodegeneration or how to prevent it.

In a previous study, we reported a correlation between post-injury accumulation of synuclein and subsequent neuronal death (Busch and Morgan, 2012). Synucleins (α-, β-, γ-) comprise a family of synaptic vesicle-associated proteins whose physiological functions are still under investigation (Bendor et al., 2013). Under normal physiological conditions, α-synuclein regulates synaptic vesicle trafficking and protects nerve terminals from degeneration (Chandra et al., 2005; Nemani et al., 2010; Busch et al., 2014; Vargas et al., 2014; Wang et al., 2014). However, α-synuclein also has deleterious roles in Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple systems atrophy, and several variants of Alzheimer's disease, collectively called synucleinopathies. In these neurological diseases, α-synuclein abnormally self-assembles into oligomers and fibrils, leading to neurotoxicity and neuronal death (Lee and Trojanowski, 2006; Beyer and Ariza, 2008; Cookson, 2009). In familial Parkinson's disease, α-synuclein aggregation

Abbreviations: DLB, dementia with Lewy bodies; MO, morpholino; PD, Parkinson's disease; RS, reticulospinal; SCI, spinal cord injury; TBI, traumatic brain injury; ThT, thioflavin T.

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and neurotoxicity result from multiplication of the α-synuclein gene or from several point mutations (Cookson, 2009; Dawson et al., 2010). Thus, there are numerous links between α-synuclein aggregation and neurodegeneration in several common diseases. Though less well-studied, deposits of β- and γ-synuclein are associated with axon pathology in PD and DLB (Galvin et al., 1999), and overexpression of a DLB-linked β-synuclein mutant or wild type γ-synuclein causes neurodegeneration and behavioral defects in animal models (Ninkina et al., 2009; Fujita et al., 2010).

In comparison, very little is known about the roles for synuclein in the injured nervous system. There is growing evidence that both SCI and traumatic brain injury (TBI) lead to increased immunoreactivity against α-, β-, and γ-synucleins in mammals, including humans (Uryu et al., 2003; Uryu et al., 2007; Sakurai et al., 2009; Mondello et al., 2013). Furthermore, α-synuclein is elevated in the cerebral spinal fluid of patients with severe TBI, and higher α-synuclein levels have been associated with worse post-injury outcomes, including a greater extent of injury and even death (Su et al., 2011; Mondello et al., 2013). However, previous studies have not determined the effects of increased synuclein levels on the neurons themselves and have not examined the aggregation state of the protein. On one hand, increased amounts of synuclein may serve positive roles by protecting nerve terminals and supporting vesicle trafficking during membrane repair and axon regeneration (Bloom and Morgan, 2011). On the other hand, increased levels of synuclein may cause its aggregation and lead to neurotoxicity.

Our previous study was the first to reveal a correlation between injury-induced synuclein accumulation and dying neurons (Busch and Morgan, 2012). We used the large, identified reticulospinal (RS) neurons of lampreys because their injury responses can be followed over time on a cell-by-cell basis. RS neurons also express high levels of synuclein mRNA, which encodes for a γ-synuclein isoform. Using this model, we showed that a predictable and reproducible subset of giant RS neurons accumulated small puncta of synuclein protein after injury, and these were the same neurons that subsequently died. Conversely, the neurons that did not accumulate synuclein after SCI were the ones that survived the injury (Busch and Morgan, 2012), which is also the same subset that undergoes robust axon regeneration (Jacobs et al., 1997). After injury, the mRNA for γ-synuclein was downregulated in all of the giant RS neurons, implying post-transcriptional mechanisms (Busch and Morgan, 2012). We therefore concluded that synuclein accumulation is a biomarker for forthcoming neurodegeneration after SCI. However, the extent to which synuclein accumulation is causal for injury-induced neurodegeneration has not yet been assessed in any experimental model, forming the premise for the current study.

Here, we demonstrate that modulating post-injury synuclein accumulation using CLR01, a small-molecule inhibitor of amyloidogenic protein aggregation (Sinha et al., 2011), or a translation-blocking synuclein morpholino, leads to clearance of excess synuclein after SCI. Reducing post-injury synuclein accumulation improves the survival of injured neurons and increases the number of axons in the spinal cord. Thus, synuclein accumulation is identified here as a novel factor contributing to injury-induced neurodegeneration.

2. Materials and methods

2.1. Spinal cord transections and drug application

Late stage larval lampreys (*Petromyzon marinus*; 10–13 cm) were anesthetized in 0.1 g/L MS-222 (Tricaine-S; Western Chemical, Inc.; Ferndale, WA). Next, the spinal cord was transected at the 5th gill, as previously described (Busch and Morgan, 2012). CLR01 (2.4 μg = 1 mM) or buffer (lamprey internal solution: 180 mM KCl, 10 mM HEPES, pH 7.4) was added at the time and site of spinal injury *via* a small piece of Gelfoam (Pfizer; New York, NY). 3′-Lissamine labeled morpholinos (10 μg; GeneTools, LLC; Philomath, OR) were applied similarly. These included a translation-blocking synuclein morpholino (Syn MO) (5′CGCGTCCATTCCTCTTTCTTTGTCT3′) generated against the start site of lamprey γ-synuclein (GenBank Accession JN544525.1) and a five base pair mismatch synuclein morpholino (MM MO) that was used as the negative control (5′CGCcTgCATTgCTCTTTgTTTcTCT3′). Afterwards, lampreys were allowed to recover at room temperature (RT) for 11 weeks. Lampreys were then re-anesthetized, and the brains and spinal cords were dissected out for further experimentation by a researcher blinded to the experimental conditions. All procedures were approved by the Institutional Animal Care and Use Committees at The University of Texas at Austin and the Marine Biological Laboratory in accordance with standards set by the National Institutes of Health.

2.2. Immunofluoresence and image analysis

Brains and spinal cords were fixed overnight in 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Immunofluoresence staining on whole mounted brains and cryosectioned spinal cords was done as previously described (Jin et al., 2009; Busch and Morgan, 2012). Briefly, the brains were labeled in whole mount with primary and secondary antibodies at 4 °C overnight, each followed by 5 × 1 h washes. Spinal cord sections were labeled with primary and secondary antibodies at RT for 1–2 h, each followed by 3 × 15 min washes. Primary antibodies used in this study included a rabbit polyclonal pan-synuclein antibody (1:100; Abcam ab6176; Cambridge, MA), a mouse monoclonal synaptotagmin 1 antibody (1:100; Developmental Studies Hybridoma Bank; DSHB mAb 48; Iowa City, IA), and a mouse monoclonal neurofilament-180 antibody (1:100; LCM16; kind gift from Dr. Michael Selzer), which have all been previously characterized in lamprey nervous tissues (Jin et al., 2009; Busch and Morgan, 2012; Lau et al., 2013). mAb 48 (asv 48) was deposited to the DSHB by L. Reichardt. For the pre-absorption experiment shown in Fig. 2D, the pan-synuclein antibody was incubated overnight at 4 °C with 100 μg/ml recombinant GST-tagged lamprey γ-synuclein before being applied to lamprey brains, and this resulted in a loss of the immunofluorescence signal. Secondary antibodies used in all experiments were AlexaFluor® 488-conjugated or AlexaFluor® 594-conjugated goat anti-rabbit or goat anti-mouse IgGs (1:300; Life Technologies; Grand Island, NY). Nuclei were stained with ProLong® Gold containing DAPI.

After immunostaining, the synuclein and synaptotagmin immunofluorescence in the cell bodies of giant RS neurons were imaged using a Zeiss LSM510 laser scanning confocal on an Axioskop 2FS upright microscope (10×, 0.3 NA Zeiss EC Plan-Neofluar objective or 40×, 0.8 NA Zeiss Achroplan water-dipping objective). Z-stacks were acquired, from which 3D projections were generated. For quantification of immunofluorescence, images were acquired under identical conditions for control and spinal-transected animals. Fluorescence intensity associated with each giant RS neuron was measured in ImageJ, followed by background subtraction using a measurement taken from the adjacent neuropil. Data from each cell type were collected and averaged from 6 to 13 lamprey brains.

NF-180 immunofluorescence and DAPI staining were imaged in spinal cord cryosections using an EVOS® FL Cell Imaging System (10×, 0.3 NA and 20×, 0.5 NA Plan-Fluorite objectives). Sections proximal, within, and distal to the lesion were sampled. For the axon analysis, all NF-180-labeled axons in the ventral half of the spinal cord were counted and averaged from 4 to 8 animals per condition.

2.3. Nissl staining and image analysis

After completing the immunofluorescence analysis, lamprey brains were Nissl-stained, as previously described (Busch and Morgan, 2012). Low-magnification images of lamprey brains were acquired either with a DFC420C camera connected to a Leica MZ10F stereoscope or an AxioCam MRc camera connected to a Zeiss SteREO Discovery V20. High-magnification images of individual M, I, and B cell regions were