Elucidating the dynamics of the neuronal stress response in driving the death of retinal ganglion cells

Madeline M. Farley, Sofia Gonzalez de Corcuera, Shufang Wang, Allison Melton, Trent A. Watkins
Department of Neurosurgery, Baylor College of Medicine, Houston, Texas
Supported by the Glaucoma Research Foundation and Mission Connect, a project of the TIRR Foundation

INTRODUCTION
The neuronal stress-responsive Dual Leucine-zipper Kinase (DLK) has emerged as an attractive drug target in neurodegenerative diseases. Inhibiting DLK is potentially neuroprotective for retinal ganglion cells (RGCs) in models of glaucoma and optic nerve injury. However, the relationship between DLK activity and neuronal apoptosis is poorly understood, as DLK activation does not acutely or necessarily result in neuronal loss and is a critical component of neuronal repair (e.g., axon regeneration).

To understand the dynamics of DLK signaling that are responsible for RGC death, we have engineered a DLK whose activity can be controlled by a small molecule. In this pilot study, we have evaluated the prospects for graded or intermittent stimulation of the stress response to probe how levels, patterns, and context of DLK activity determine the fates of RGCs.

DESIGN & METHODS
Optic nerve crush results in retrograde DLK signaling. The normally labile DLK protein is stabilized near the site of RGC axon injury, resulting in its oligomerization. Thus activated, DLK mediates retrograde stress signaling to the RGC nucleus, detected by IHC for the phosphorylated form of the transcription factor c-Jun. DLK knockdown, provides proteolysis and neuronal protection of RGCs after optic nerve crush.

STIMULATION OF THE NEURONAL STRESS RESPONSE IN RGCs IN VIVO
Controlled stimulation of the neuronal stress response. To allow for graded and dynamic stimulation of DLK stress signaling, we have engineered a version whose stability, and therefore activity, is controlled by a blood-brain barrier permeant small molecule antibiotic, trimethoprim (TMP). This construct consists of the active domain of DLK, an epitope tag for easy detection (Flag), and 1-2 engineered versions of the bacterial dithiobis(succinimidyl) dithiadiazole (DHFR) that confer instability to the protein, leading to targeting to the proteasome in the absence of TMP.

The engineered versions of DLK harboring either a single C-terminal DHFR destabilizing domain ("ddDLK") or both C-terminal and N-terminal DHFR destabilizing domains ("ddFKBP DLK") are introduced by transient transfection in vitro to HEK293T cells (for testing and optimization) or by intravitreal AAV2-mediated transduction into young adult C57Bl6 mice to RGCs in vivo (for testing effects on neuronal apoptosis and axon regeneration).

IN VITRO OPTIMIZATION OF STRESS RESPONSE ACTIVATION BY A SMALL MOLECULE
Dynamic control of DLK, Destabilized DLK (ddDLK), but not yellow fluorescent protein (YFP), results in dose-dependent DLK signaling (p-c-Jun) in response to TMP in transfected 293T cells. Washout of TMP reduces this signaling.

Improved background and dynamic range by adding a second DHFR destabilization domain. Drug-induced DLK signaling was detected by Western blot of 293T cell lysates for the Flag-tagged destabilized DLK constructs and the downstream transcription factor phospho-c-Jun.

CONCLUSIONS
Low background activity and superior dynamic range provided by adding a second DHFR destabilizing domain represent valuable improvements on the original ddDLK design.

Regulated expression of ddDLK as a tool for neuronal stress and axon regeneration.

Unregulated overexpression of ddDLK results in the rapid loss of RGCs.

Dynamic control of DLK, Destabilized DLK (ddDLK), but not yellow fluorescent protein (YFP), results in dose-dependent DLK signaling (p-c-Jun) in response to TMP in transfected 293T cells. Washout of TMP reduces this signaling.

Improved background and dynamic range by adding a second DHFR destabilization domain. Drug-induced DLK signaling was detected by Western blot of 293T cell lysates for the Flag-tagged destabilized DLK constructs and the downstream transcription factor phospho-c-Jun.

NEXT STEPS
Alternative destabilizing domains & drugs. The use of destabilizing FKBP domains generates opportunities for stimulating DLK with different time courses and dynamics based on the distinct pharmacokinetic properties of their stabilities. The small molecule Shield1.

Improving RGC axon regeneration. Preliminary data suggest that stimulating DLK signaling improves growth of PTEN-deficient RGCs following optic nerve crush, without resulting in greater RGC apoptosis (Caspase-3). TMP was dosed one day prior to ON crush injury, and sprouting axons were assessed 3 days after crush.

ACKNOWLEDGEMENTS
This work is supported by the Glaucoma Research Foundation Shaffer Grant and by Mission Connect, a project of the TIRR Foundation. Madeline Farley is also supported by NEI Training Grant T32EY00701. Valuable support and advice provided by Matt Rasband, Preethi Somasundaram, and Mary Edgington.