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

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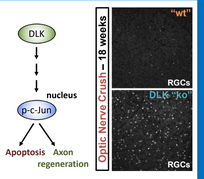
INTRODUCTION

The neuronal stress-responsive Dual Leucine-zipper Kinase (DLK) has emerged as an attractive drug target in neurodegenerative diseases. Inhibiting DLK is potently 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).

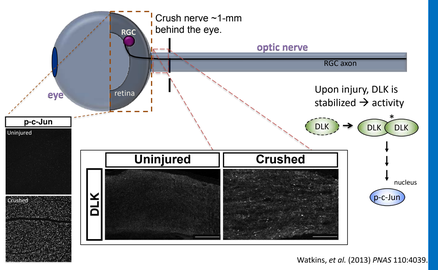
To understand the dynamics of DLK signaling that are responsible for RGC death, we have engineered a DLK whose activity can be controlled by a small molecule. In this pilot study, we have evaluated the prospects for graded or intermittent stimulation of the stress response to probe how levels, patterns,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



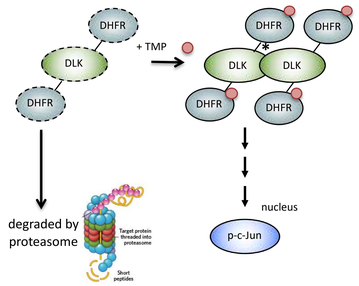
DLK mediates stress signaling by phosphorylation of the transcription factor c-Jun. *DLK knockout provides prolonged neuroprotection of RGCs after optic nerve crush.*

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 nuclei, detected by IHC for the phosphorylated form of the transcription factor c-Jun. This signaling persists until the slow and steady apoptosis of the large majority of the RGCs over the subsequent weeks and months.*



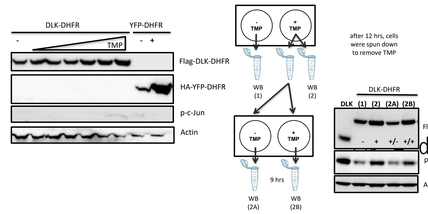
Watkins, *et al.* (2013) *PNAS* 110:4039.



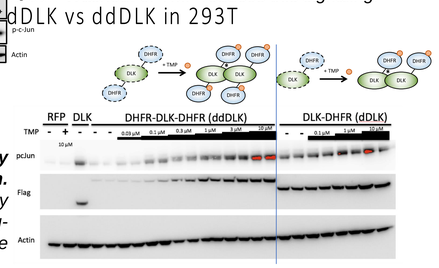
Controlled stimulation of the neuronal stress response. *To allow for graded and dynamic stimulation of DLK stress signaling, we have engineered a version whose stability, and therefore activity, is controlled by a blood-brain barrier permeant small molecule antibiotic, trimethoprim (TMP). This construct consists of the active domain of DLK, an epitope tag for easy detection (Flag), and 1-2 engineered versions of the bacterial dihydrofolate reductase (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 ("dDLK") or both C-terminal and N-terminal DHFR destabilizing domains ("ddDLK") 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

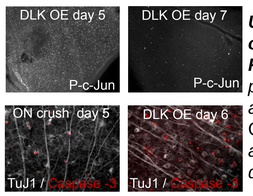


Dynamic control of DLK signaling. *Destabilized DLK (dDLK), but not yellow fluorescent protein (YFP), results in dose-dependent DLK signaling (p-c-Jun) in response to TMP in transfected 293T cells. Washout of TMP reduces this signaling.*

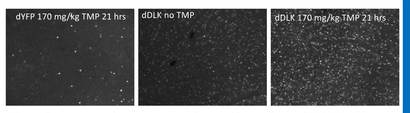


Improved background and dynamic range by adding a second DHFR destabilization domain. *Drug-induced DLK signaling was detected by Western blot of 293T cell lysates for the Flag-tagged destabilized DLK constructs and the downstream transcription factor phospho-c-Jun.*

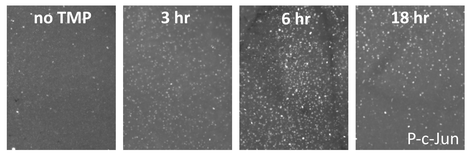
STIMULATION OF THE NEURONAL STRESS RESPONSE IN RGCs *IN VIVO*



Unregulated DLK overexpression kills RGCs. *DLK overexpression results in apoptosis* (activated Caspase 3, red) *in the absence of DHFR destabilizing domains.*

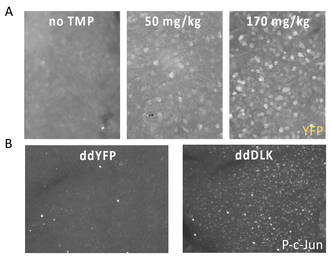


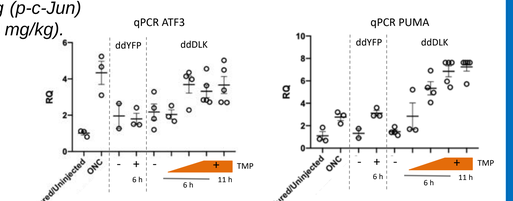
Background stress signaling by dDLK. *Even in the absence of TMP, a DLK with a single DHFR destabilizing domain leads to low level phospho-c-Jun in RGCs (whole-mount retina).*



Pharmacodynamics of DLK signaling following stimulation of ddDLK in RGCs by systemic delivery of TMP. *Drug-induced DLK signaling was detected by immunostaining whole-mount retinas for the downstream transcription factor phospho-c-Jun.*

In vivo responsiveness to double-destabilized constructs in mouse retina. *(A) ddYFP exhibits dose-responsiveness. (B). ddDLK signaling (p-c-Jun) 6 hrs after i.p. injection of TMP-lactate (170 mg/kg).*





Stimulation of pro-regenerative and pro-apoptotic transcription in RGCs. *TMP-induced DLK signaling, like optic nerve crush (ONC), upregulates expression of both pro-regenerative Atf3 and pro-apoptotic Puma (qPCR of retina).*

CONCLUSIONS

TECHNICAL ADVANCES

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

Destabilized DLK constructs allow for dynamic control of DLK signaling by small molecules for studies to determine the thresholds for RGC death and repair.

RGC DEATH AND AXON REGENERATION

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

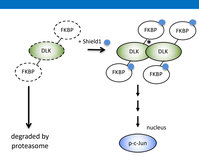
Controlled stimulation of the neuronal stress response by TMP-mediated ddDLK activation results in upregulation of both pro-apoptotic and pro-regenerative gene expression.

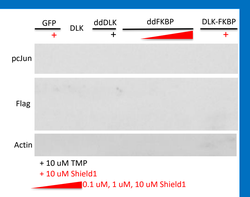
Prior stimulation of the neuronal stress response by ddDLK does not appear to result in greater apoptosis of RGCs after optic nerve damage.

Stimulation of the neuronal stress response by ddDLK may facilitate improved RGC axon regeneration enabled by knockout of the tumor suppressor PTEN.

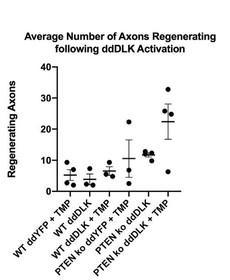
NEXT STEPS

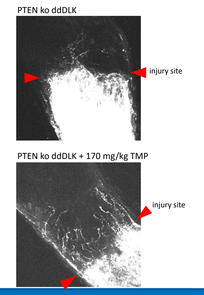
Alternative destabilizing domains & drugs. *The use of destabilizing FKBP domains generates opportunities for stimulating DLK with different time courses and dynamics based on the distinct pharmacokinetic properties of its stabilizer, the small molecule Shield1.*

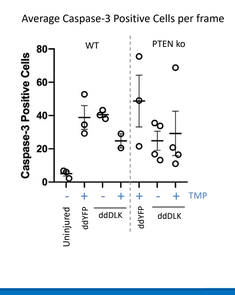




Improving RGC axon regeneration. *Preliminary data suggest that stimulating DLK signaling improves growth of PTEN-deficient RGCs following optic nerve crush, without resulting in greater RGC apoptosis (Caspase-3). TMP was dosed one day prior to ON crush injury, and sprouting axons were assessed 3 days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







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