Activation of Slack potassium channels (KCNT1) triggers an increase in protein maturation

T. J. Malone[1], P. Licznerski[2], E. A. Jonas[2], L. K. Kaczmarek[1,3]

[1]Dept. of Cell. and Mol. Physiology, [2]Intrnl. Medicine/Section Endocrinol., [3]Pharmacology, Yale School of Medicine, New Haven, CT

Abstract

The Slack ion channel is a member of a family of large conductance sodium-activated potassium channels. Several gain-of-function Slack mutations are associated with early-onset epilepsy and severe intellectual disability in humans. Misregulation of protein synthesis is a possible explanation for the intellectual disability phenotype. FMRP, an important regulator of mRNA translation, binds both Slack mRNA and the Slack protein. The association of Slack with FMRP stimulates channel activity, raising the possibility that activation of Slack channels may also regulate the function of FMRP. Our laboratory has previously identified Slack as required for a protein synthesis-dependent recovery from an extended period of inhibition in Aplysia neurons following stimulation, further suggesting that Slack may play a role in the regulation of protein expression. Using FRAP experiments to visualize real-time protein expression in a stable Slack-expressing HEK cell line and in mouse cortical neurons, together with pharmacological manipulation and silencing RNA knockdowns, we have found that Slack activation causes an increase in the levels of the reporter protein. We also found that the R455H Slack mutation, one of the clinical mutations affecting human patients, causes a baseline increase in levels of the reporter protein. This mechanism of Slack-dependent reporter synthesis potentially represents the first instance of the direct modulation of protein synthesis by activation of an ion channel. The increased synthesis caused by the Slack mutation indicates that misregulation of mRNA translation or protein maturation is a possible mechanism to explain the intellectual disability experienced by patients with Slack mutations.

Background

Gain-of-function Slack mutations

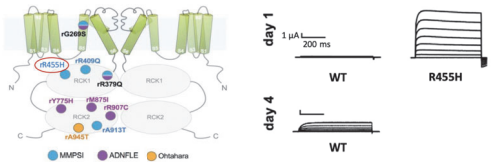

Figure 1: Schematic diagram of two Slack subunits with locations of epilepsy syndrome mutations (left). Positions of the mutations within the C-terminal "gating ring" containing two RCK domains (Regulators of Conductance for K, RCK1 and RCK2) are based on X-ray structure. Representative current readout from whole cell patch clamp from oocytes injected with WT or mutant Slack. (right)

Slack-dependent protein synthesis

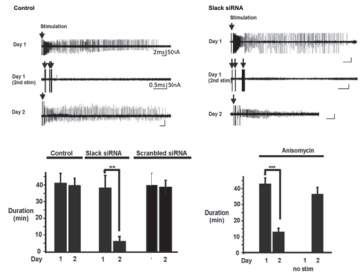

Figure 2: In Aplysia bag cell neurons, Slack is required for a protein synthesis-dependent recovery from extended inhibition.

Results

Dendra2 FRAP monitors protein synthesis

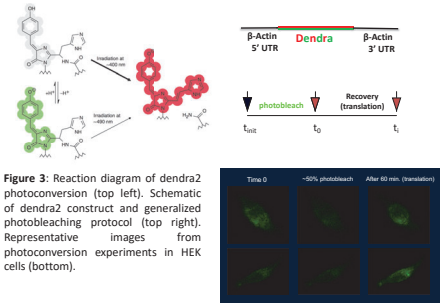

Figure 3: Reaction diagram of dendra2 photoconversion (top left). Schematic of dendra2 construct and generalized photobleaching protocol (top right). Representative images from photoconversion experiments in HEK cells (bottom).

Slack activation causes increased fluorescence recovery

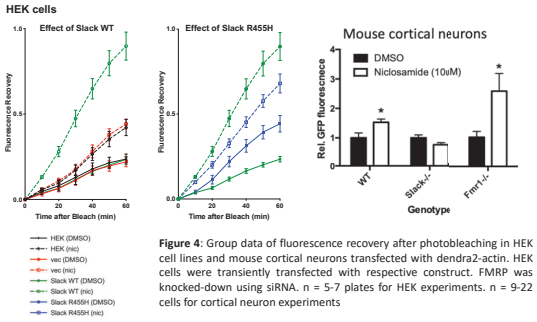

Figure 4: Group data of fluorescence recovery after photobleaching in HEK cell lines and mouse cortical neurons transfected with dendra2-actin. HEK cells were transiently transfected with respective construct. FMRP was knocked-down using siRNA. n = 5-7 plates for HEK experiments. n = 9-22 cells for cortical neuron experiments

Protein maturation and mRNA translation are likely causes of increased fluorescence recovery

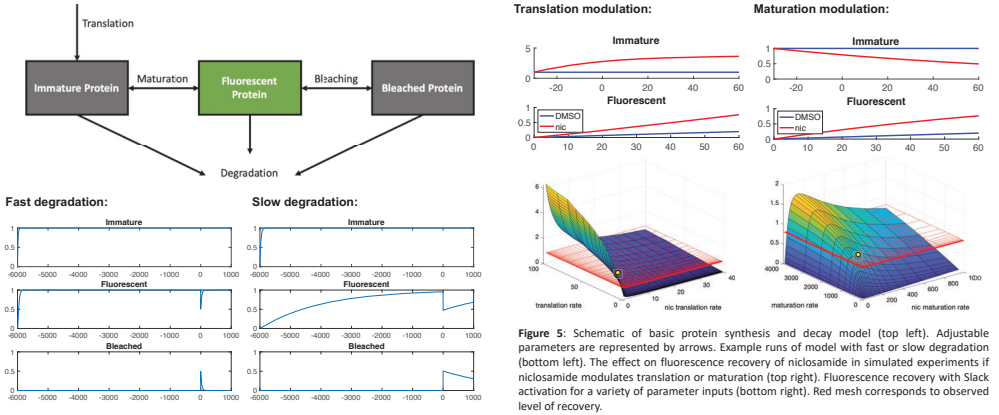

Figure 5: Schematic of basic protein synthesis and decay model (top left). Adjustable parameters are represented by arrows. Example runs of model with fast or slow degradation (bottom left). The effect on fluorescence recovery of niclosamide in simulated experiments if niclosamide modulates translation or maturation (top right). Fluorescence recovery with Slack activation for a variety of parameter inputs (bottom right). Red mesh corresponds to observed level of recovery.

Slack 455H mutation increases dendra protein levels

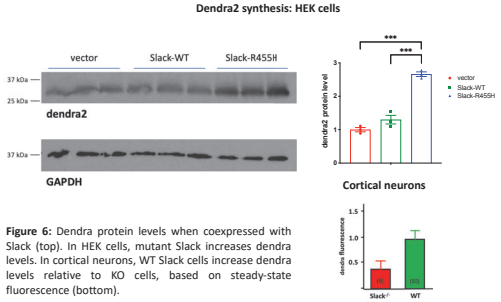

Figure 6: Dendra protein levels when coexpressed with Slack (top). In HEK cells, mutant Slack increases dendra levels. In cortical neurons, WT Slack cells increase dendra levels relative to KO cells, based on steady-state fluorescence (bottom).

Slack activation causes higher fluorescence increases in the absence of bleaching

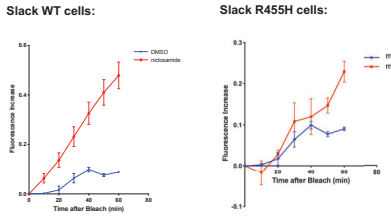

Figure 7: FRAP-like experiments where no photobleaching is performed. The spontaneous fluorescence increase during experiment is higher in the presence of Slack WT cells than in control cells (left). Preliminary data suggests spontaneous fluorescence increase is higher in Slack R455H cells than in Slack WT cells.

Increased fluorescence recovery is not caused by new dendra translation

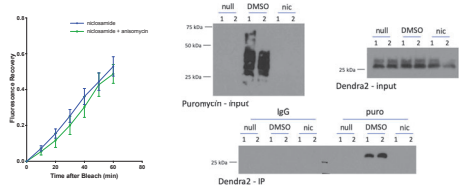

Figure 8: FRAP experiment in presence of a translation inhibitor does not change the effect of Slack (left). Niclosamide alone inhibits global translation (top right). A puromycin co-IP performed after puromycin incorporation, shows dendra2 translation is inhibited by niclosamide (bottom right).

Intracellular pH changes cannot explain increased fluorescence recovery

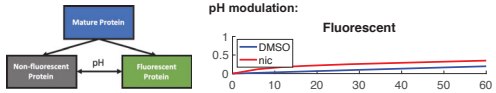

Figure 9: About 85% of dendra protein is fluorescent at physiological pH. A shift to 100% fluorescence due to pH changes would be insufficient to explain observed fluorescence increases with niclosamide. FRAP experiments using Hibernate-A media to better control intracellular pH will be performed in the future.

Conclusions/Future Directions

- **Slack activation with niclosamide causes increased fluorescence recovery of the dendra2-actin reporter construct**
- **Increased fluorescence recovery is likely due to an increase in the rate of protein maturation or release from the ribosome**
- **The Slack R455H mutation spontaneously causes this effect in the absence of an activator**

Future directions include:

- Exploring how Slack is able to effect modulate fluorescence recovery
  - Is effect Slack specific or is potassium flux sufficient?
  - Is the C-terminus of Slack necessary and/or sufficient?
- Looking for direct evidence that protein maturation is responsible for observed fluorescence changes
  - Perform ribosome pulldown with WT or mutant Slack
  - Create alternate dendra constructs to determine causal locus (UTR, coding region, etc.)

References

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Acknowledgments

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