TRAPping Fear-Based Proteomic Changes in Activated Neurons Using Targeted Recombination

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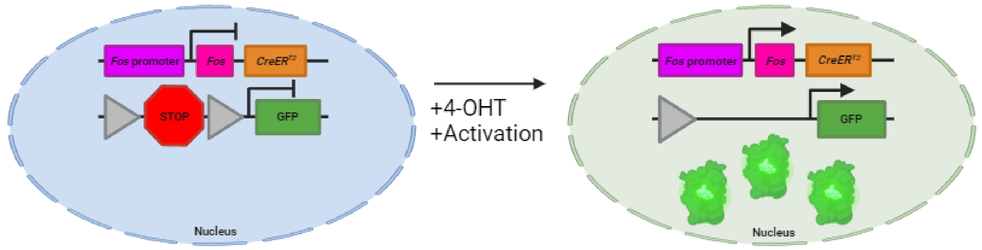
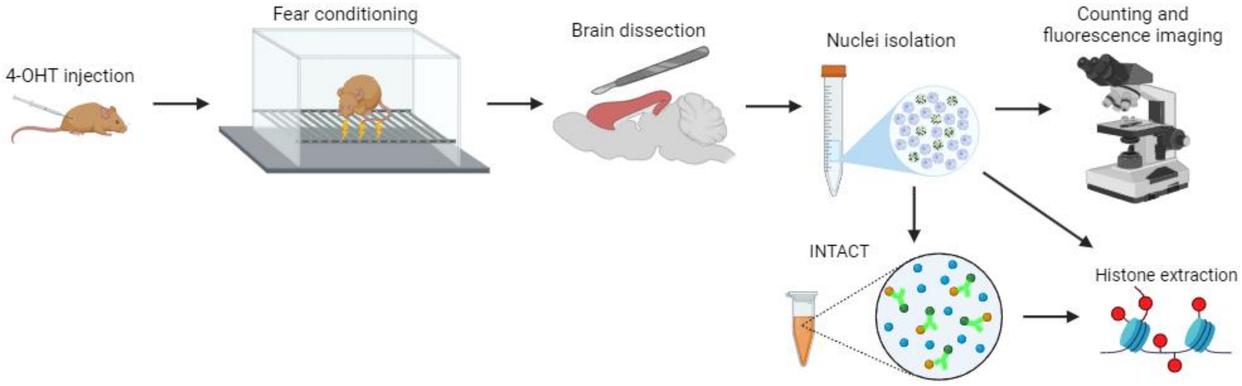
Funded by Grants for Faculty Mentoring Undergraduate Research

Background for Experiment 1: TRAPping Mice

This projects aims to uncover the biological mechanisms of PTSD, a psychiatric disorder that affects over 16 million people in the US and is characterized by debilitating cognitive symptoms such as anxiety, depression, and mood instability in response to a traumatic event. To evaluate epigenetic signatures of fear memory formation in the small subset of neurons that are activated by stimuli, we plan to use TRAP (targeted recombination in active populations) to permanently label activated neurons with GFP. Expression of the gene for CreERT2, a Cre recombinase modified to bind to tamoxifen, is placed under control of the promoter for Fos, a gene transiently expressed when a neuron fires an action potential. Wildtype mice don't express Cre, so we use transgenic mice containing the Fos-Cre and Sun1-GFP genes. If we inject a precursor of tamoxifen known as 4-hydroxytamoxifen (4-OHT) right before an animal is fear conditioned, neuronal activity plus tamoxifen will trigger production of CreERT2 and allow it to excise a transcription stop signal gene flanked by two loxP sites, which then induces expression of a Sun1-GFP gene, a fusion GFP that is localized to the nucleus. Thus, only activated neurons can be permanently labeled in their nuclei, and we can control when this labeling starts by our time of injecting 4-OHT. Once these nuclei are marked, we can extract histones from them to analyze changes in the epigenome of neurons activated by fear.



Protocol for Experiment 1



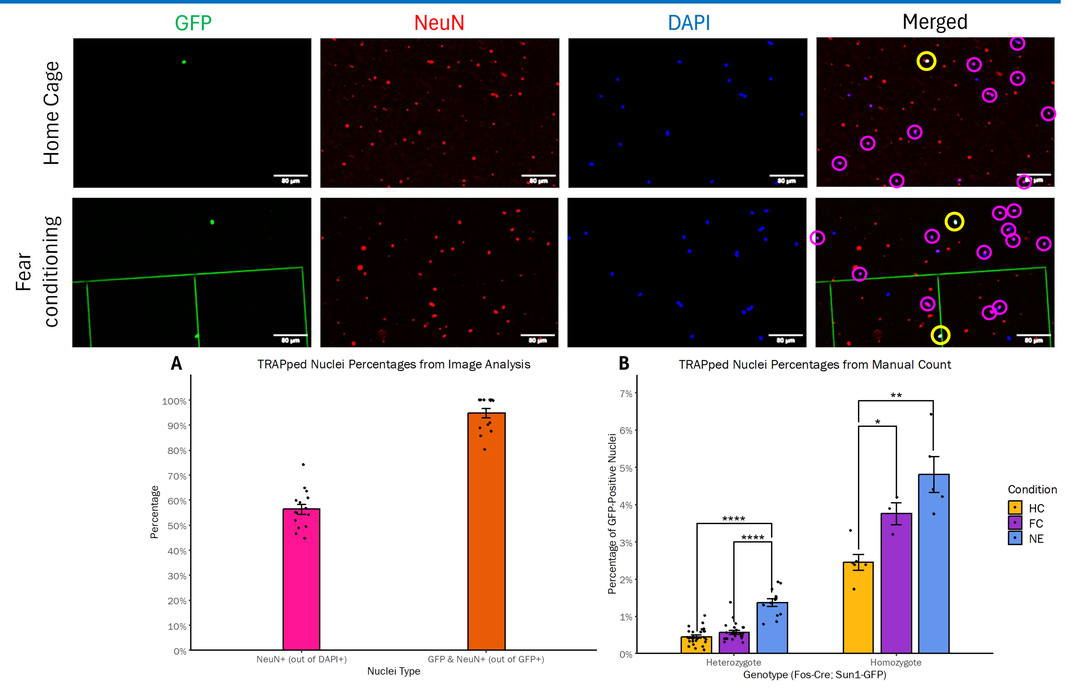
1. Inject TRAP mice with 4-OHT and subject to fear conditioning
2. Dissect mice and extract cerebral cortex, amygdala, hippocampus, and cerebellum
3. Isolate nuclei with density gradient
4. Manual count and fluorescence imaging of nuclei
5. Purify GFP+ nuclei with INTACT
6. Extract histones from isolated and purified nuclei

Results for Experiment 1

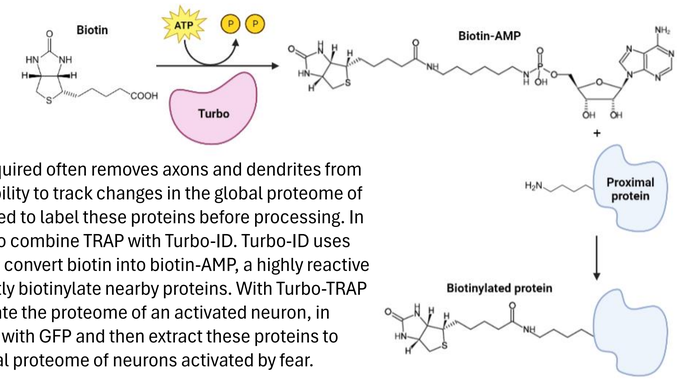
Image Analysis: 56% of the nuclei are NeuN+, 93% of the GFP+ nuclei are also NeuN+ (Fig. A)
Manual Count:

- Heterozygotes showed no significant increase in GFP+ nuclei after fear conditioning but the novel environment mice showed a significant increase compared to control and fear conditioned mice (Fig. B)
- Homozygotes showed a significant increase in GFP+ nuclei after fear conditioning and novel environment exposure, compared to the control (Fig. B)

Takeaways:
The nearly 100% percentage of GFP- and NeuN-positive nuclei out of all GFP-positive nuclei indicates that GFP production in TRAP mice only occurs in neurons and is localized to their nucleus, which confirms that the TRAP method works. The significantly higher percentage of GFP-positive nuclei in fear conditioned and novel environment homozygous mice compared to control, and in novel environment heterozygous mice compared to control indicates that neuronal activation was higher than normal in those mice. The non-significant difference between percentage of GFP-positive nuclei in control and fear conditioned heterozygous mice was unexpected and could be explained by lower overall rates of TRAPping in heterozygous mice.



Background for Experiment 2: Turbo Neurons

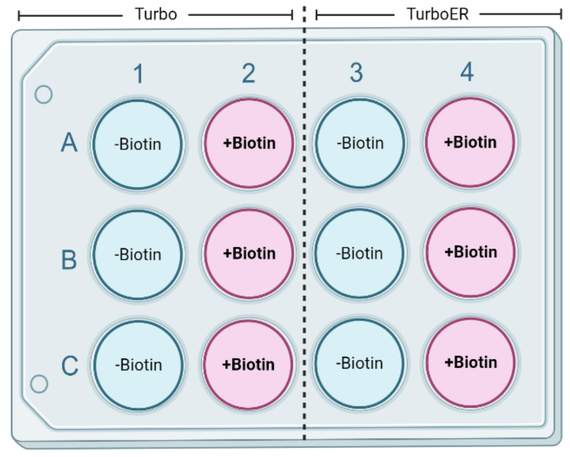


Normal tissue processing required often removes axons and dendrites from neurons, complicating our ability to track changes in the global proteome of activated neurons. So, we need to label these proteins before processing. In pursuit of this goal, we plan to combine TRAP with Turbo-ID. Turbo-ID uses the biotin ligase Turbo, which convert biotin into biotin-AMP, a highly reactive intermediate, so it can robustly biotinylate nearby proteins. With Turbo-TRAP we can permanently biotinylate the proteome of an activated neuron, in place of labeling the nucleus with GFP and then extract these proteins to examine changes in the global proteome of neurons activated by fear.

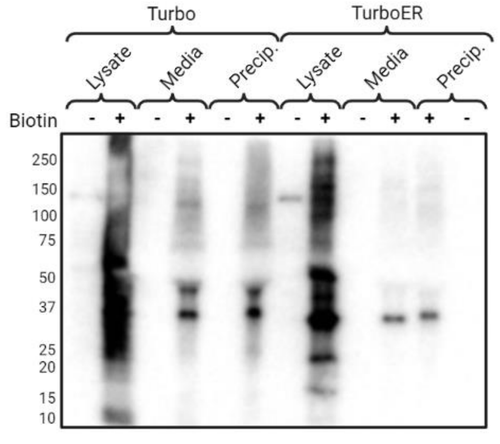
Protocol for Experiment 2

In vivo experiments with Turbo-TRAP require transgenic mice that express Turbo after recombination by CreERT2. While we wait for these mice to be generated, we are first conducting this experiment in N2As to confirm that Turbo works effectively.

1. Deliver the Turbo or TurboER gene to half of the N2As using viral transfection; TurboER is a form of Turbo that localizes to the ER lumen
2. Treat half of N2As with 5 mM biotin
3. Collect whole protein lysate, media, and protein precipitate from media
4. Western blot using an HRP-conjugated streptavidin probe to confirm biotinylation.



Results for Experiment 2



- For both types of N2A, the lysates show the highest levels of biotinylation across several proteins for the biotin-supplemented cells
- Media and precipitates for Turbo N2As show similar increase in biotin compared to control
- Media and precipitates for TurboER N2As had very little biotin
- All lanes showed a high concentration of biotin around 35 kDa

The high concentration of biotin makes sense because Turbo enzyme is dispersed throughout the neuron and catalyzes robust biotinylation of nearby proteins.
The decrease in biotin concentration in media and precipitate from the TurboER N2As, compared to the Turbo N2As, could be explained by the localization of TurboER to the ER lumen, which would lead to less biotinylation of proteins far from the ER that are secreted from the cells.
The high concentration of biotin around 35 kDa in lysate, media, and precipitate for both Turbo and TurboER N2As could be Turbo or TurboER, since it is roughly the same size that we would expect the enzyme to be.