Testing the Role of Calcitonin Gene-Related Peptide (CGRP) in Scar Formation after Multiple Tissue Injury

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Introduction

Optimal healing for a traumatic wound or injury is described as completely regenerating the damaged tissue. This study focuses on replacing what the nerve secretes that promotes nerve and tissue regeneration, more specifically in the military population. A study with a sample size of 4,563 soldiers deployed in Iraq and Afghanistan found that 82% of these soldiers suffered musculoskeletal injuries due to explosions or blasts. Out of these injuries, 94% of them resulted in amputations. However, amputations are not the desired outcome of these injuries, and the question becomes: is there a specific protocol that the body should undergo to promote tissue repair? Amputations occur when there is severe and excessive tissue damage and dysfunction, therefore, if more tissue was salvageable, there would be better surgery outcomes. The goal of this study is to develop treatment that promotes regeneration rather than scar tissue formation.

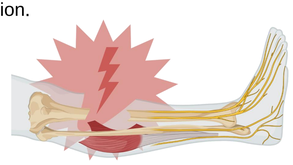


Figure 1: This model shows what occurs to a human leg when impacted by an explosion injury. Multiple tissues are injured including bone, muscle, skin, and nerves

The African Spiny Mouse (*Acomys cahirinus*) is an example of a mammal that has excellent regeneration properties like lizards and starfish. Often, these rodents along with the common lab mouse (*Mus musculus*), are used as a comparative mammalian model to study the mechanisms of regeneration by comparing their regeneration and scar-forming abilities, respectively (Figure 2).

Previous RNA sequencing reveals that several neurotrophic factors are upregulated during scar formation in *Mus* and downregulated or unchanged during regeneration in *Acomys* (Figure 3). One notable neuropeptide, the Calcitonin Gene Related Peptide (CGRP), is not expressed in regeneration in *Acomys* but expressed in scar formation in *Mus*. CGRP is a known component of pain transduction and migraine onset, but there is still uncertainty around its role in wound healing and scar formation. **This study evaluates the direct role CGRP plays in scar formation.**

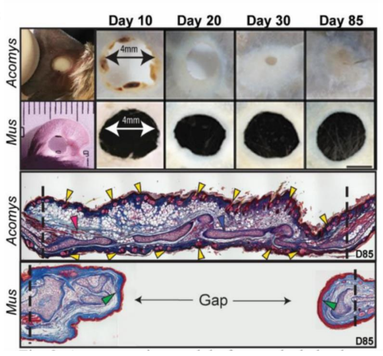


Figure 2: Depicts an 85-day progression of regeneration and scar formation in the *Acomys cahirinus* and *Mus musculus* following a 4mm biopsy ear punch.

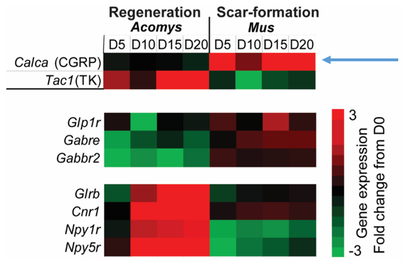
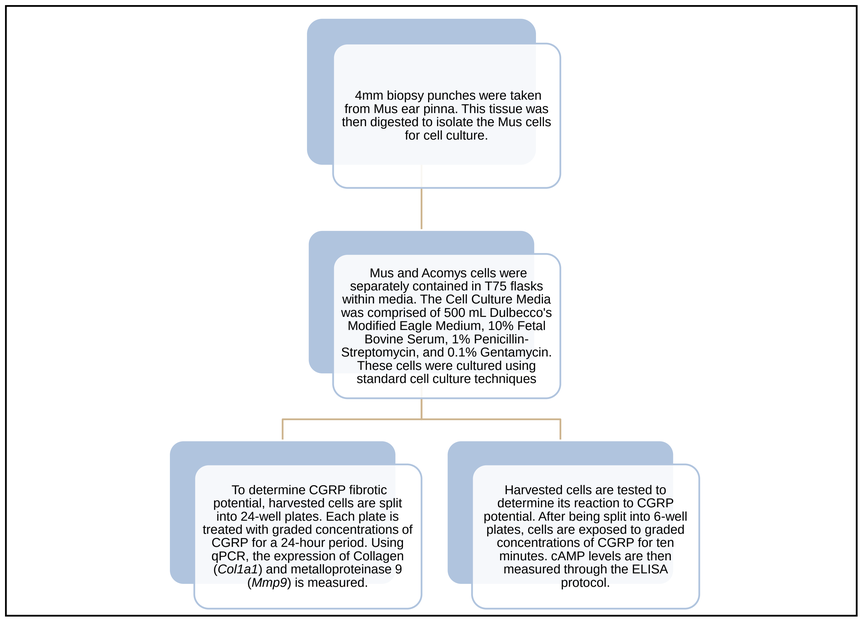


Figure 3: This RNAseq comparison shows certain pro-fibrotic and pro-regenerative neurotransmitters are expressed following injury. CGRP is clearly shown as a neuropeptide that is barely, if at all, upregulated in regeneration in *Acomys*, but highly expressed in scar formation in *Mus*.

Based on previous research, it can be hypothesized that CGRP can promote tissue fibrosis following injury. To test this, we can expose *Acomys* cells to CGRP and measure its scar-forming potential. One indicator of scar-formation is an increase in collagen production. Therefore, to measure fibrotic potential of CGRP we expose *Acomys* and *Mus* cells to exogenous CGRP, collect the RNA after 24 hours of exposure and measure changes in collagen expression using qPCR.

Methods



Results

Figure 4: *Acomys* and *Mus* cells were treated with different concentrations of CGRP (0.1 ug/mL, 1 ug/mL, 5 ug/mL, and No Treatment) for a 24-hour period. After the incubation period, the cells are prepared for qPCR following the SYBR Green qPCR Protocol. The primers used in the first qPCR run was *Col1a1* and *B2m* (housekeeping gene). The second qPCR run used the *Mmp9* primers and *B2m*. The plate was run for 40 cycles.

Figure 5: To determine if cells were responding directly to CGRP, we measured levels of downstream mediators. This pathway demonstrates the intracellular signaling pathways of CGRP receptor activation. When CGRP binds to CGRP receptors, one effect of the activation is cAMP level elevation from Adenylate Cyclase. Therefore, if cells are responsive to CGRP we should observe a rise in cAMP levels.

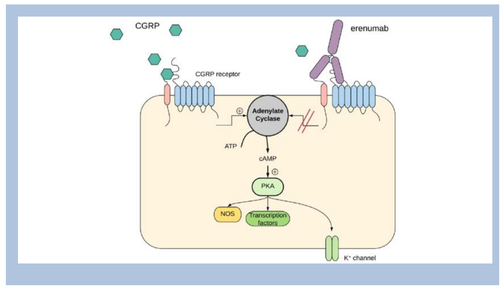
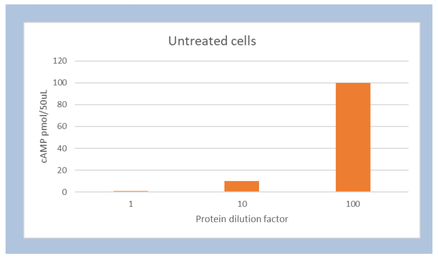
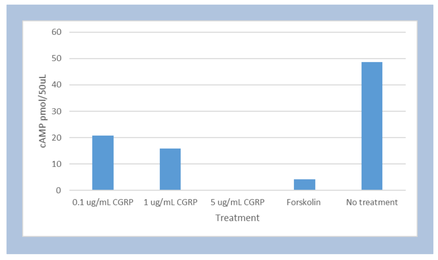


Figure 6: To measure cAMP levels we use an ELISA. cAMP levels were measured for each concentration of 10 minutes of CGRP treatment (0.1 ug/mL, 1 ug/mL, and 5 ug/mL), no CGRP treatment, and Forskolin treatment (positive control). We observed higher cAMP levels in the no treated cells compared to our positive control. To better understand our assay results, we diluted protein levels 1:1 concentration, 1:10 concentration, and 1:100 concentration. cAMP measurements increased with increasing concentrations suggesting there was something in the sample that interfered with the assay's ability to accurately measure cAMP.





Discussion

Reaction to CGRP:

- *Acomys* cells exposed to 1 ug/mL showed the highest expression increase in *Col1a1* compared to untreated controls.
- *Mus* cells showed no change in *Col1a1* expression, no matter the concentration of CGRP.
- *Acomys* cells exposed to 0.1 ug/mL and 5 ug/mL of CGRP show a potential increase in *Mmp9* expression.
- *Mus* cells show a decrease in *Mmp9* expression with all concentrations of CGRP.
- More replicates need to be run to determine if these trends are accurate.

Determining profibrotic potential:

- Because the cAMP assay was inaccurate, we cannot fully determine if the cells were responding directly to CGRP. Therefore our conclusions on the qPCR results are still preliminary.

Conclusions & Further Research

Results suggests:

- If the above trends hold true, it is possible CGRP is increasing *Col1a1* expression in *Acomys* cells which would suggest potential fibrotic activity.

Further Research tactics:

- Investigate profibrotic potential further → replicating cAMP assay procedure to determine if cells are responsive to CGRP.
- Working with more cells (BCA assay recommends 1000 ug/mL of protein to get consistent cAMP results, more cells = more protein)
- Treat cells with CGRP for longer than 10 minutes
- Cleaning protein protocol to prevent interfering substances
- Performing Western blot for different CGRP downstream mediators (ex. pCREB)

References

Farkas, J. E., & Monaghan, J. R. (2017, April 10). A Brief History of the Study of Nerve Dependent Regeneration. *Neurogenesis*, *4*(1), 1-12. https://doi.org/10.1080%2F23262133.2017.1302216

Gawriluk, T. R., Simkin, J., Thompson, K. L., Biswas, S. K., Clare-Salzler, Z., Kimani, J. M., Kiama, S. G., Smith, J. J., Ezenwa, V. O., & Seifert, A. W. (2016, April 25). Comparative analysis of ear-hole closure identifies epimorphic regeneration as a discrete trait in mammals. *Nature Communications*, *7*(1), 1-16. https://doi.org/10.1038%2Fncomms11164

Liu, X., Sun, S. Q., Hassid, A., & Ostrom, R. S. (2006). cAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and Smad signaling in cardiac fibroblasts. *Mol Pharmacol*, *70*(6), 1992-2003. https://doi.org/10.1124/mol.106.028951

Schmittgen, T. D., & Livak, K. J. (2008, June 5). Analyzing Real-Time PCR data by comparative Ct Method. *Nature Protocols*, *3*(6), 1101-1108. doi:10.1038/nprot.2008.73

Seifert, A. W., Kiama, S. G., Seifert, M. G., Goheen, J. R., Palmer, T. M., & Maden, M. (2012). Skin shedding and tissue regeneration in African spiny mice (Acomys). *Nature*, *489*(1), 561-565. doi:10.1038/nature11499

Seifert, A. W., & Muneoka, K. (2018, January 15). The blastema and epimorphic regeneration in mammals. *Developmental Biology*, *433*(2), 190-199. doi:10.1016/j.ydbio.2017.08.007

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