# Engineering 3D Skeletal Muscle Primed for Neuromuscular Regeneration Following Volumetric Muscle Loss

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**Statement of Purpose:** Volumetric muscle loss (VML) is a skeletal muscle defect that overwhelms native regenerative capabilities. Current treatments are limited and fail to fully recover lost function. Previous tissue engineered muscle constructs have had varied success but remain limited in their ability to encourage neuromuscular regeneration. Others have shown that exercise encourages neural infiltration[1] and that the protein agrin induces acetylcholine receptor (AChR) clustering in myoblasts[2]. In the current study we have assessed the separate effects of exercise post-VML on neural regeneration and of agrin application (soluble and chemically tethered) on AChR clustering in our constructs, and will evaluate their synergistic effects in ongoing studies (**Fig. 1**).

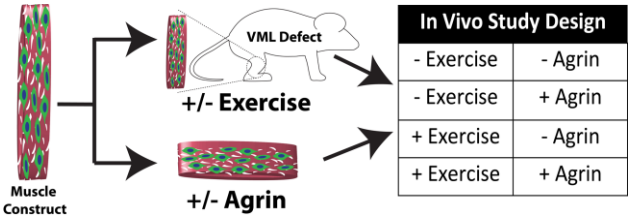


**Figure 1.** Experimental design investigating the separate and synergistic effects of exercise post-VML and *in vitro* agrin pre-treatment on neuromuscular regeneration.

**Methods:** Electrospun hydrogels were fabricated[3–5], seeded with C2C12 myoblasts, and implanted into murine VML defects in 8 mice[3]. Following a 2-week recovery, 4 mice began a treadmill regimen at 12m/min[1]. After 3 weeks of exercise, samples were assessed for muscle regeneration and neural infiltration via histology. For *in vitro* studies, agrin was chemically conjugated to the scaffold surface at 2, 10, and 50 µg/ml using the EDC crosslinker. C2C12s were seeded and cultured for 7 days. Soluble agrin at 5 or 50 ng/ml was included in the culture media of day 6 C2C12-seeded unconjugated scaffolds for 24 or 48 hours. Samples were stained for myosin heavy chain (MHC), α-bungarotoxin (αBTX) for AChR clusters, and β3-tubulin (β3T) for neurofilament. We quantified AChR cluster coverage.

**Results:** VML defects in exercised mice contained previously unseen levels of neurofilament and some AChR clusters deep within the regenerating defect which was not seen in non-exercised mice (**Fig. 2**). Soluble and tethered agrin increased AChR cluster coverage and length compared to controls. Control clusters were located primarily ~15 µm above the scaffold surface while constructs treated with soluble agrin for 48 hours had a broad distribution peaking at ~30 µm above the surface. Interestingly, tethered agrin resulted in a shift in cluster density towards the 10 µm location with a second small peak at 30 µm (**Fig. 3**).

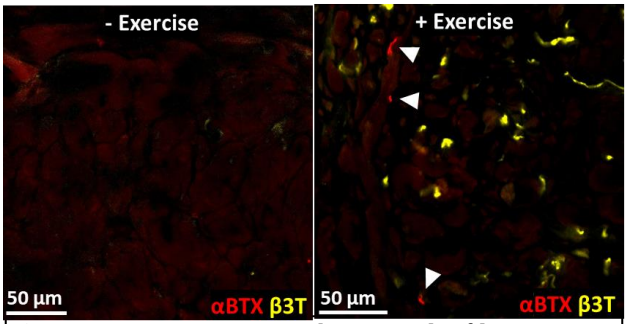


**Figure 2.** Exercise increases host neural infiltration (β3T; yellow) and AChR clusters (arrows: αBTX; red) within VML defects. Both images are within defect area.

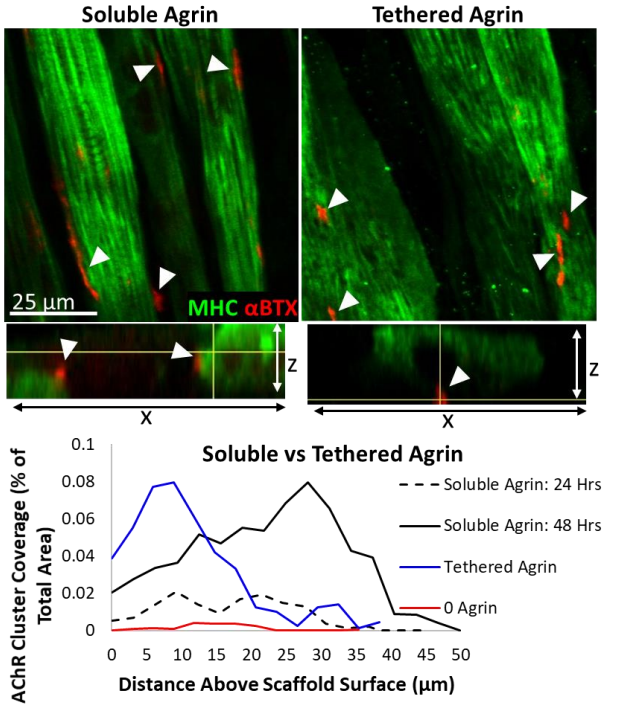


**Figure 3.** Soluble and tethered agrin induce more AChR cluster coverage than controls. Tethered agrin induces narrower distribution localized mainly ~10 µm above the scaffold while clusters from soluble agrin were broadly distributed peaking at ~30 µm above the scaffold. Arrows: AChR clusters on myotubes.

**Conclusions:** Therapeutic exercise encourages robust neural infiltration to regenerating muscle post-VML with some AChR clusters. Constructs pre-treated with soluble and tethered agrin result in drastically increased AChR clustering with interesting differences in cluster location between groups. Ongoing studies will compare the synergistic effects of exercise and pre-treated constructs to enhance NMJ formation.

**References:** 1. Quarta M. Nat Comm. 2017; 8:15613. 2. Bruneau EG. Dev. Biol. 2005; 288(1):248-258. 3. Gilbert-Honick J. Biomaterials. 2018; 164:70-79. 4. Zhang S. Biomaterials. 2014; 35(10):3243-3251. 5. Gilbert-Honick J. Cell Transpl. 2018;1-13.