12 Month TECHNICAL PROGRESS REPORT

SCIRF #*2015 I-03*
PI: *Stephen Tomlinson, PhD (originally Rick Schnellmann, PhD).*
Grant Title: *Formoterol, an FDA-approved drug, stimulates mitochondrial biogenesis as a novel therapeutic strategy for spinal cord injury.*
18-month Technical Progress Report

This report includes data generated over the 18 month period. A manuscript is in the final stages of preparation.

Aim 1: *In Progress.*

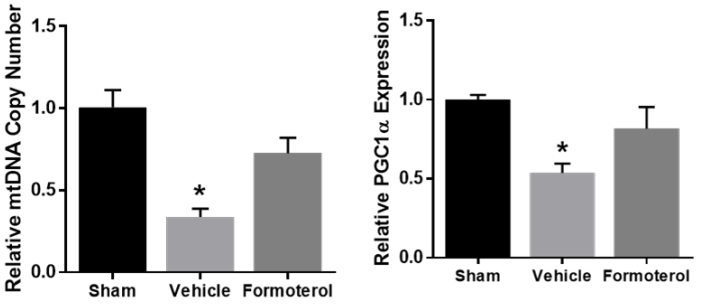

Figure 1. mtDNA content (*A*) and PGC1α mRNA expression (*B*) following daily formoterol treatment (0.1 mg/kg, i.p) on MB 7 days post-SCI. (* *p < 0.05 by Student's t-test compared to Sham*). Data reported as mean ± SEM, *n*=3-6 per group.

Specific Aim 1: Determine the effect of optimized formoterol-induced MB on functional recovery in mouse model of SCI.

Female C57/BL6 mice were subject to SCI using a force-controlled pneumatic impactor-induced contusion model. **Using this method, mice experienced sustained mitochondrial dysfunction for at least 7 days, as evidenced by decreased mitochondrial DNA copy number and PGC1α expression in the injury site compared to sham controls (Figure 1, sham v vehicle).** For preliminary studies, mice received daily i.p. injections of 0.1 mg/kg formoterol or vehicle, beginning one hour post-SCI and continuing for up to 15 days. **At 7 days post-SCI, mtDNA copy number and PGC1α expression of formoterol-treated animals was near that of sham mice, indicating enhanced mitochondrial biogenesis in the spinal cord of this treatment group (Figure 1, sham v formoterol).** Assessment of motor function was performed one day post-SCI, and every second day thereafter, using the Basso-Mouse Scale (BMS). Formoterol-treated mice exhibited higher BMS scores compared to vehicle controls beginning at Day 7, indicating improved functional recovery. By Day 15, the BMS score of formoterol-treated SCI mice was near double that of vehicle-treated animals (4 v 2; Figure 2).

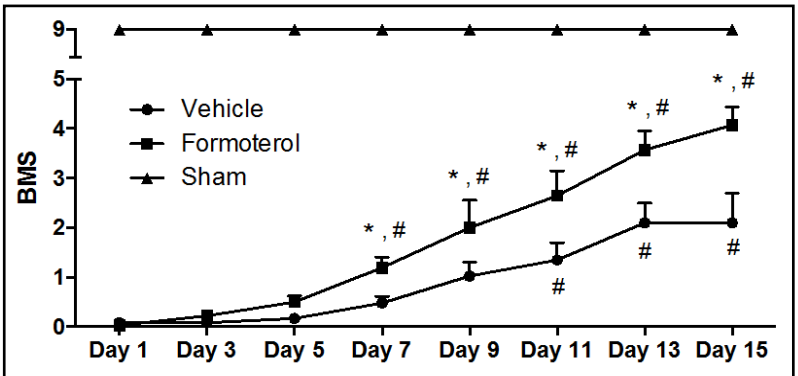

Figure 2. BMS assessment depicting recovery of motor function following SCI and daily injections of 0.1 mg/kg formoterol (squares) or vehicle (circles). As expected, sham mice displayed no loss of function (triangles), retaining a BMS score of 9 throughout the experiment. * *p<0.05 compared to Vehicle; # p<0.05 compared to Day 1 by One-Way ANOVA followed by Tukey's post-hoc test.*

A subset of mice were euthanized on Day 7 and spinal cord tissue was collected for qPCR analysis of mitochondrial DNA copy number and various mitochondrial-

and nuclear-encoded mitochondrial genes**, as well as protein analysis**. Although preliminary analysis of the peri-injury site using a limited number of animals (*n*=3-6/group) revealed no differences in copy number or any of the genes examined, data are trending towards increased expression of mitochondrial genes in formoterol- compared to vehicle-treated mice (Figure 2). **Protein analysis of nuclear-encoded mitochondrial genes in the peri-injury site 7 days post-SCI (*n*=3-5/group) indicated no differences in PGC1α or ATP Synthase β between vehicle and formoterol-treated animals, similar to that observed with mRNA analysis. However, NDUFS1 protein was increased in the peri-injury site of formoterol-treated SCI mice compared to vehicle-treated SCI mice (Figure 4A, B). Interestingly, immunoblot analysis also revealed formoterol-induced differences in various mitochondrial biogenesis signaling proteins after 7 days of treatment. ERK phosphorylation was increased 2-fold in the peri-injury site of formoterol-treated SCI mice compared to that of both sham and vehicle-treated SCI mice, while FOX01a phosphorylation was decreased in the formoterol-treated mice. No difference was observed in phosporylated AKT, although expression was trending up in the formoterol-treated animals. More analysis needs to be completed to determine the implications of these changes.**

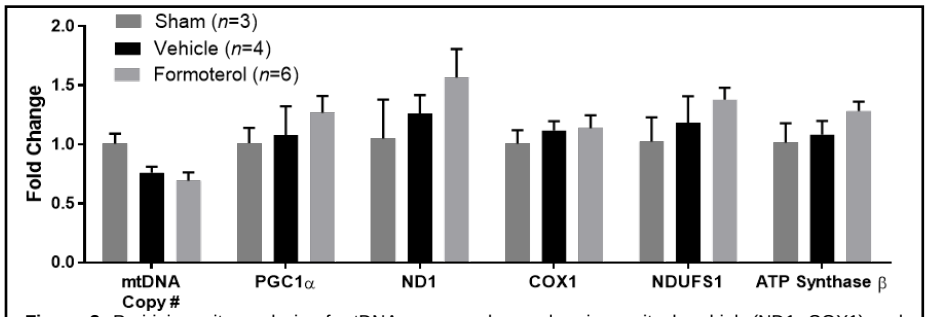

Figure 3. Peri-injury site analysis of mtDNA copy number and various mitochondrial- (ND1, COX1) and nuclear-encoded (PGC1α, NDUFS1, ATP Syn β) mitochondrial genes 7 days following SCI with daily i.p injections of 0.1 mg/kg formoterol or vehicle. *mtDNA Copy #: mitochondrial DNA copy number; PGC1α: peroxisome proliferator-activated receptor γ coactivator 1α; ND1: NADH dehydrogenase 1; NDUFS1: NADH:ubiquinone oxidoreductase core subunit S1; COX1: cytochrome c oxidase 1; ATP Synase β: ATP synthase subunit β*

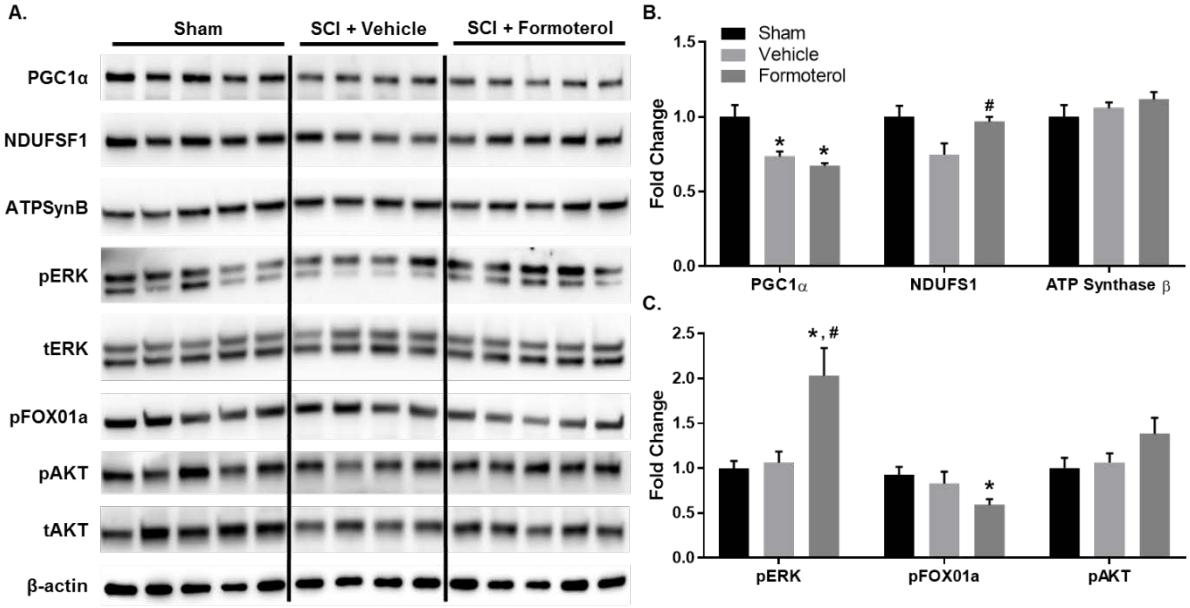

Figure 4. Peri-injury site analysis of nuclear-encoded mitochondrial proteins (***A, B***) and MB signaling-associated proteins (***A, C***) 7 days following SCI with daily i.p injections of 0.1 mg/kg formoterol or vehicle. ** p<0.05 compared to Sham, # p<0.05 compared to Vehicle by One-Way ANOVA followed by Tukey's post-hoc test*

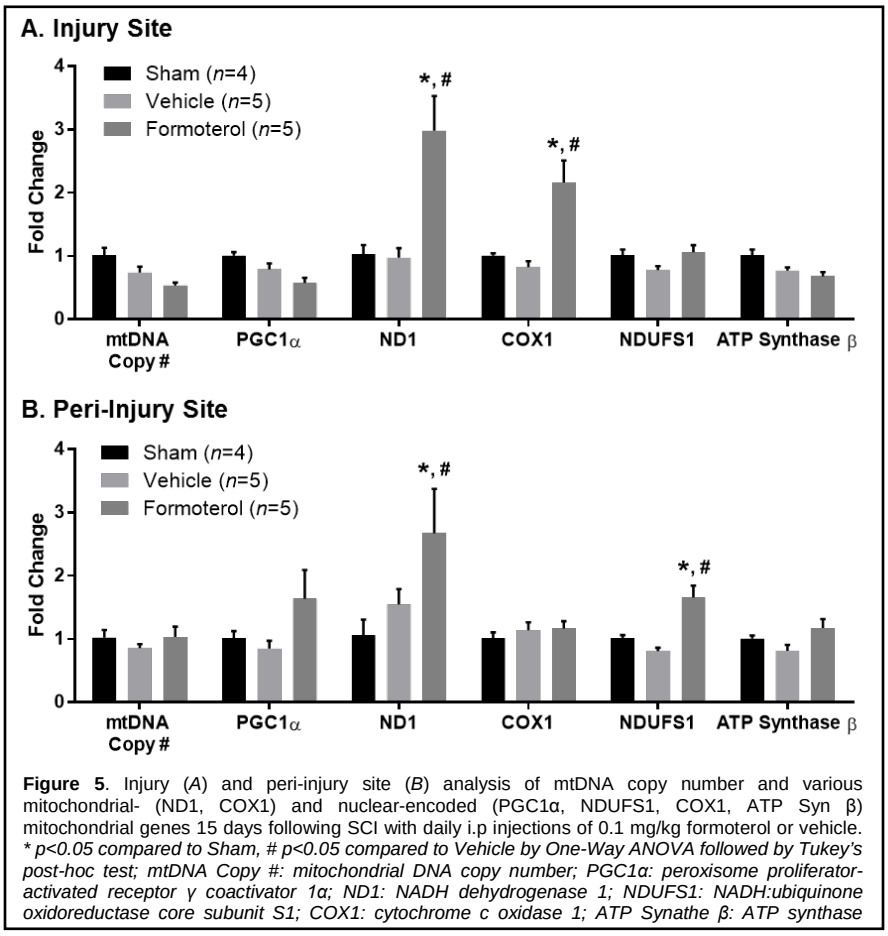

Figure 5. Injury (*A*) and peri-injury site (*B*) analysis of mtDNA copy number and various mitochondrial- (ND1, COX1) and nuclear-encoded (PGC1α, NDUFS1, COX1, ATP Syn β) mitochondrial genes 15 days following SCI with daily i.p injections of 0.1 mg/kg formoterol or vehicle. ** p<0.05 compared to Sham, # p<0.05 compared to Vehicle by One-Way ANOVA followed by Tukey's post-hoc test; mtDNA Copy #: mitochondrial DNA copy number; PGC1α: peroxisome proliferator-activated receptor γ coactivator 1α; ND1: NADH dehydrogenase 1; NDUFS1: NADH:ubiquinone oxidoreductase core subunit S1; COX1: cytochrome c oxidase 1; ATP Synathe β: ATP synthase*

However, mice exposed to daily 0.1 mg/kg formoterol treatment for 15 days following SCI, exhibited increases in various mitochondrial genes in the injury site and peri-injury site. ND1 and COX1, two mitochondrial-encoded genes, increased 3-fold and 2-fold, respectively, in the injury site of formoterol-treated mice compared to vehicle. In the peri-injury site, mice exposed to formoterol exhibited a 2-fold increase in ND1 and NDUFS1, a nuclear-encoded mitochondrial gene, compared to vehicle, and data for PGC1α and ATP Synthase β trended towards increased expression. It should be noted that a limited number of animals were used for this analysis (*n*=4-5/group; Figure 5).

Mice were also euthanized 3 days post-SCI and the spinal cord extracted for histopathological analysis. At this time, distinct differences in histology were observed between groups. The sham animals maintained a visible central canal and dorsal/ventral horns, as well as a clear anterior median fissure. Vehicle-treated SCI mice depicted a loss of white matter and the central canal and anterior median fissure were difficult to observe. Formoterol-treated SCI mice, however, showed distinctly less loss of white matter than the vehicle group. Further, the above morphological

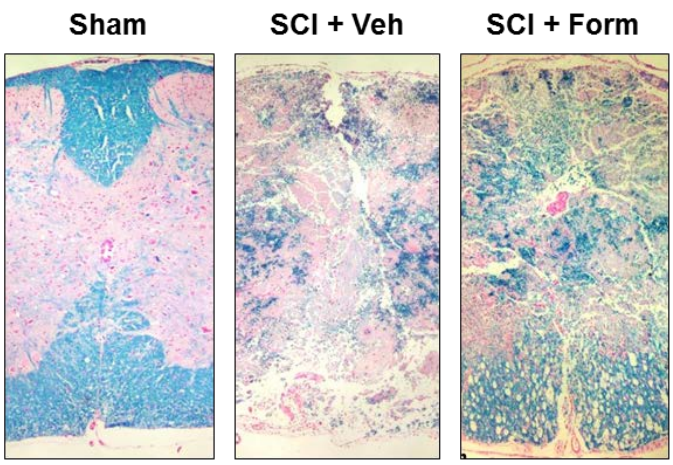

Figure 6. Transverse histophatological analysis of the spinal cord of sham and SCI mice following 3 days of daily i.p injections of vehicle or 0.1 mg/kg formoterol using Luxol Fast Blue stain, which stains myelin (white matter) blue.

characteristics were more easily visible (Figure 6).

To discern the optimal dose of formoterol for induction of mitochondrial biogenesis in the spinal cord, naïve female C57bl/6 mice were treated with either 0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg or vehicle for 15 days, then the spinal cord was extracted for analysis of mitochondrial DNA copy number. A significant increase in mtDNA was observed in the spinal cords of mice exposed to 0.3 mg/kg, which was not observed with the other doses (Figure 7).

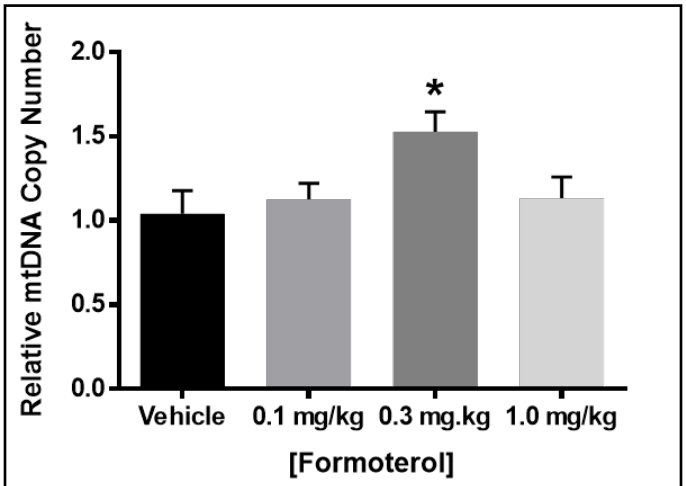

Figure 7. Analysis of mitochondrial DNA copy number following 15 days of daily i.p injections of various concentrations of formoterol or vehicle. * *p<0.05 compared to Vehicle by Student's t-test.*

Aim 2: *In process*

Specific Aim 2: Determine the effect of optimized formoterol administration on vascular recovery within spinal cord and blood spinal cord barrier function following SCI in mouse.

List all articles, publications, presentations, grant applications or grant awards related to the SCIRF award.

An abstract entitled "Mitochondrial biogenesis as a novel therapeutic strategy for spinal cord injury" was presented as a poster at SfN in San Diego, CA in November 2016.

An abstract entitled "Enhanced mitochondrial biogenesis for the treatment of spinal cord injury" will be presented as a poster at Experimental Biology in Chicago, IL in April 2017 and is a finalist for the Division of Neuropharmacology Postdoctoral Scientist Award and for the Delores Shockley Poster Award. Winners will be determined at EB.

We anticipate submitting a manuscript for publication in March.

We anticipate a VA merit grant submission in the September 2017 cycle.