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Neural Stem Cell Metabolic Changes in the Presence of Interferon-gamma

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Introduction

The spinal cord serves as the major connection between the brain and other nerves distributed throughout the body. Injury to spinal cord tissue can be caused by genetic disorders, infections, and trauma that results in a loss of connectivity that leads to clinical disability. Repair of the injured spinal cord is difficult because the CNS has limited regenerative capacity[1]. One therapeutic option for spinal cord injury is the replacement of damaged cells by stem cells[1]. Neural stem cells (NSCs) are defined as cells in the human nervous system that can develop into any type of neuron or glial cell after forming an intermediate precursor cell[2]. Previous work has identified the cytokine interferon-gamma (IFN-γ) as a stimulatory molecule that promotes the differentiation of NSCs to mature neurons[3]. One potential method to expose NSCs to IFN-γ is to seed them onto a biocompatible hydrogel composed of modified chitosan (**Figure 1).**

In this experiment, two scientific questions will be explored: What specific metabolic pathways are activated to guide neural stem cell differentiation after NSCs are exposed to IFN-γ? Is oxidative phosphorylation used by neural stem cells as an energy requirement after they are exposed to IFN-γ? In order to examine the metabolism of NSCs, global metabolomic profiling and immunohistochemistry will be used to track biochemical changes and protein migrations in the cells as they are exposed to IFN-γ[5,6].

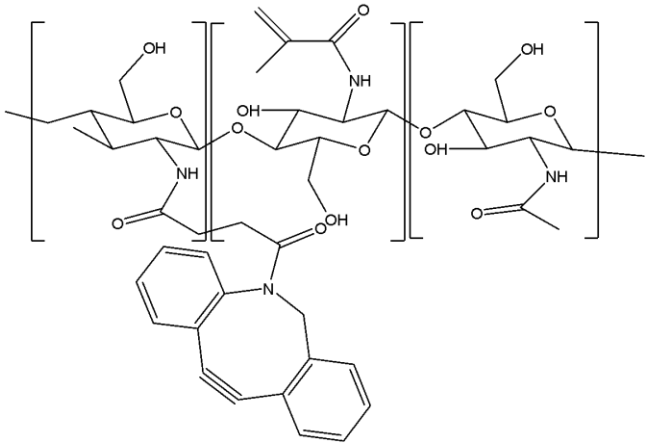

Figure 1: Structure of methacrylamide chitosan-bibenzocyclooctyne (MAC-DIBO) hydrogel used in the immunohistochemistry portion of this experiment[4].

There are multiple methods used to analyze small molecule metabolites in tissues in either a targeted or untargeted manner. Targeted metabolomics involves using an already-existing knowledge of specific biochemical metabolites to quantitatively measure and identify specific metabolites, while global metabolomics seeks to quantitatively measure and identify metabolites without the pre-existing knowledge of specific metabolites[7]. Metabolomic analysis utilizes chromatography and mass spectrometry to separate and detect metabolites. In hydrophilic interaction liquid chromatography, a small molecule interacts with the polar stationary phase that is run through a column. Compounds of varying degrees of polarity are subsequently eluted by changing the composition of the mobile phase running through the column[8]. After separation, metabolites are introduced into a mass spectrometer as ions via the source. A common technique for producing ions of biological molecules is electrospray ionization (ESI). In this method ions are produced when analytes flow through a charged capillary. This removes solvent and introduces a charge[9]. After the small, ionized particles are formed, they travel through a magnetic field and are deflected by a magnet. This deflection is measured after the particles contact a detector that is located at the end of the mass spectrometer. After the metabolites are separated via HILIC, a mass spectrometer can be used so their mass to charge ratio and fragmentation pattern can be recorded and compared to a known data of fragmentations. By comparing data, scientists are able to see which metabolite the unknown molecule could be and then compare the unknown molecule's molecular weight to a

molecular weight calculated by the Fiehn Calculator. This step will allow scientists to confirm that they have the molecule that they have been looking for.

Immunohistochemistry will be used to identify key metabolites present in the neurons, neural stem cells, and their mitochondria. After fixing the cells in hydrogel structures and onto microscope slides, they will be stained with antibodies directed against specific proteins. In this experiment, all NSC nuclei will be stained with DAPI. Staining the nuclei will allow us to examine the presence of NSCs and staining the three different types of cells will allow us to determine the phenotypes of NSCs that correlate with metabolic changes.

Neural stem cell research has the potential to identify new therapeutic modalities to treat the damaged spinal cord. This experiment will provide a biochemical signature of tissue regeneration. By studying the effects of IFN-γ on neural stem cells, it opens the potential to manipulate metabolism to generate new central nervous system tissue for site specific repair of injury.

Materials and Methods

Metabolite Extraction and Mass Spectrometry Analysis

Cultured Neural Stem Cells (NSCs) were incubated with soluble IFN-γ, immobilized IFN-γ, and no IFN-γ for twenty-four hours. After incubation with and without the IFN-γ, the metabolites were extracted and analyzed based on a modified method from Taraboletti et al.[10]. Using HPLC grade chloroform, methanol, and water, metabolites were lysed and extracted from NSCs (based on a modified version of the Bligh and Dyer liquid/liquid extraction method[11]). Extracted metabolites were placed into a CentriVap Concentrator (LABCONCO, Kansas, MO) to be dried before storing for later analysis in the mass spectrometer. Before injection into the hydrophilic interaction liquid chromatography (HILC) column (Luna 3 µNH2 100 Å, 150 mm x 1.0 mm, Phenomenex, Torrance, CA) on the Micro200 LC machine (Eksigent, Redwood, CA), metabolites had to be suspended in 35% acetonitrile. Suspended metabolites were loaded into the Micro200 LC machine, along with water and acetonitrile mobile phases both adjusted to a pH of 7.3, and ran at a 30µL/min gradient. Separation occurred in the following format: 98% B at 0 minutes, 95% B at 1 minute, 80% B at 5 minutes, 46% B at 6 minutes, 14.7% B at 13 minutes, 0% B at 17 minutes, 100% B at 17.1 minutes, and 100% B at 23 minutes. Metabolites were separated as they interacted with the polar mobile phases and then they were placed into a Sciex 5600 + TripleTOF mass spectrometer to be analyzed. The nebulizer gas, heater gas, and curtain gas pressures were set to 15 psi, 20 psi, and 25 psi, respectively, on the mass spectrometer and then the NSC metabolites were ionized at a voltage of 5000 V. Metabolite mass to charge ratio (*m/z*) fragmentations were identified in positive mode after setting the spectrometer settings to analyze with Information Dependent Acquisition (IDA) for untargeted metabolites with a 60-1000 Da time-of flight scan and a 10 counts/s background threshold. A collision energy spread of 20 V was also used during *m/z* ratio fragmentation identification[10].

Metabolite Fragmentation Identification

Metabolite mass to charge ratio fragmentations were aligned in the mass spectrometer by their retention time and mass, which allowed the significantly dysregulated (abnormal) features to be identified. Dysregulated featured metabolites were plotted so that their fragmentations could be graphically compared between the soluble, immobilized, and no IFN-γ treatments. After the treatment fragmentations were compared, dysregulated featured metabolites were identified and then the Fiehn Calculator[12] was used to calculate the molecular weight of known metabolites with an H^+, K^+, Na^+, and NH_4^+ adduct added onto it. Once the appropriate metabolite molecular weights were calculated, the unknown metabolite fragmentations from the mass spectrometer were examined again to match their molecular weights to the calculated molecular weights from the Fiehn calculator. Human Metabolomics Data Base (HMDB)[13] was used to examine known fragmentation patterns of known metabolites whose molecular weights matched the Fiehn calculator. After examining the fragmentation patterns of known metabolites, unknown fragmentation patterns from the mass spectrometer were compared, using PeakView, so that the unknown metabolites could be confirmed as an actual metabolite compound. Confirmed metabolites were statistically compared to determine if IFN-γ expression differed between the soluble, immobilized, and missing IFN-γ treatments.

Immunohistochemistry

Neural stem cells were seeded into a MAC-DIBO hydrogel (See **Figure 1** for MAC-DIBO structure.) and subcutaneously incubated in adult rats. After incubation, the hydrogels were extracted and paraffinized until they were ready to be stained. Before the slides could be stained, they had to be deparaffinized and rehydrated. For the first step of the deparaffinization/re-hydration method, 100%, 95%, and 70% concentrated ethanol was made. Once each concentration of ethanol was made, beakers were filled with xylene, 100% ethanol, 95% ethanol, 70% ethanol, and tap water and placed in the following order: xylene, xylene, 100% ethanol, 100% ethanol, 95% ethanol, 70% ethanol, and tap water. After the beakers were filled enough with each liquid to cover the slides and placed into the correct order, the slides were placed into a slide holder and then into the first container of xylene for 5 minutes. After those first 5 minutes, then the slides were placed into the second beaker of xylene for 5 minutes. Then, the slides were placed into the 100% ethanol beakers, 95% ethanol beaker, and 70% ethanol beaker for 3 minutes each. After rinsing the slides in tap water, they were hydrated with phosphate buffer saline (PBS) solution until they were stained. Following deparaffinization/re-hydration, the tissues on each slide were stained with DAPI. Stained tissue slides were examined under an Olympus IX81 inverted fluorescent microscope system with a Hamamatsu ORCA-R2 high resolution cooled digital CCD camera set to Phase contrast/bright-field of DAPI, GFP, CY3, CY5 for the presence of DAPI cells. Slides were examined for the presence of scaffolds that had any NSCs present on them.

Results and Discussions:

The purpose of this study is to determine if overall metabolism is altered in differentiating NSCs. In order to answer this question, the NSCs seeded onto MAC hydrogels were incubated with soluble IFN-γ, immobilized IFN-γ, or no IFN-γ. After twenty-four hours of

treatment, a modified version of the Bligh and Dyer extraction technique was performed. The Bligh and Dyer extraction technique was used to extract polar and nonpolar metabolites from NSC samples. Polar metabolites were subsequently separated using a HILC column. Following separation, analytes were analyzed by ESI-MS to determine their identity and chemical properties. For each metabolite, the *m/z* values were collected for both parent ions and associated fragments generated by collision induced dissociation. Qualitative differences in metabolite levels were subsequently compared through multivariate statistical analysis (**Figure 2**). When one looks at **Figure 2**, they can see that the metabolic profile of the three treatment groups is unique. The clustering of sample treatment groups indicates that there is a difference in metabolism from NSCs when incubated with soluble IFN-γ, insoluble IFN-γ, or no IFN-γ at all.

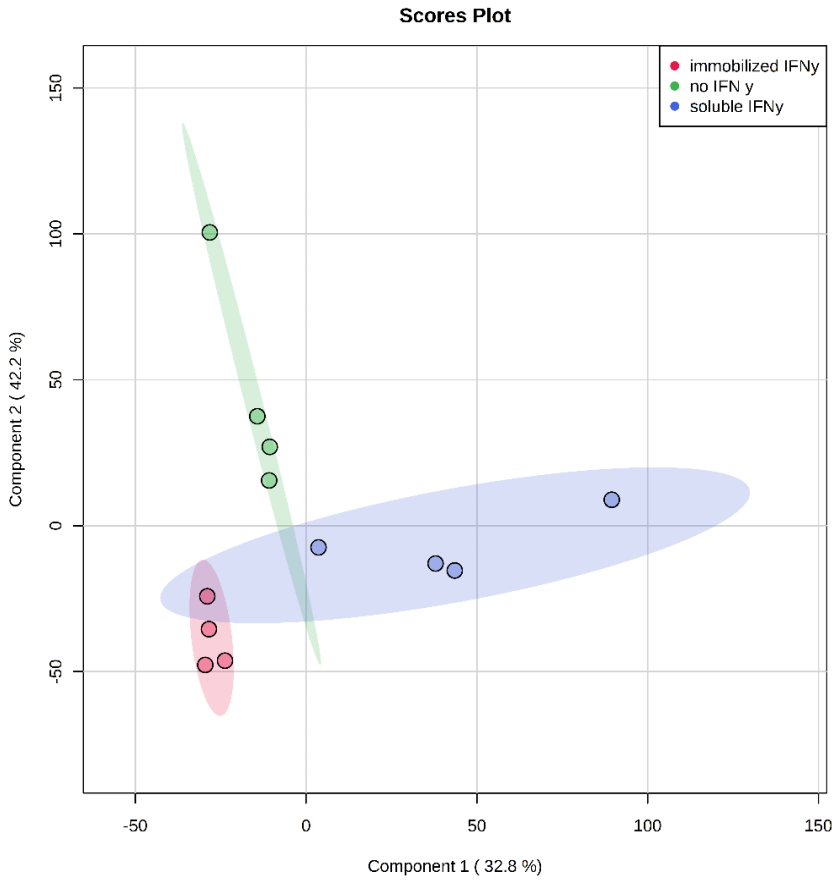

Figure 2: This principal component analysis plot shows graphical comparison of mass spectrometer fragmentations between the NSCs treated with soluble IFN-γ, insoluble IFN-γ, and no IFN-γ at all.

In order to determine what metabolites were differentially regulated in NSCs from each treatment, the Fiehn calculator was used to determine molecular weights of known metabolites with adducts on them. Once the metabolites' molecular weights were determined, the Human Metabolomics Data Base was used to find known mass spectrometer fragmentations of known metabolites. After mass spectrometer data was found, it was compared to the raw mass spectrometer data that was obtained from the Sciex 5600 + TripleTOF mass spectrometer. During comparison, PeakView was utilized to show the raw data so that the fragmentations could be compared to the known fragmentation displayed on the Human Metabolomics Data Base website. After searching the website and comparing fragmentations to the raw data, tryptophan was finally matched (**Figure 3**, **Figure 4**,). The fragmentations match at mass to charge ratios of 130, 140, and 160 with

intensities of 19, 17, and 37, respectively (**Figure 3**, **Figure 4**). **Figure 4** also serves to help conclude that the mass fragmentations between the known and unknown samples were the same. After the intensities were matched, the Fiehn molecular weights were examined again to confirm that the metabolite found was tryptophan.

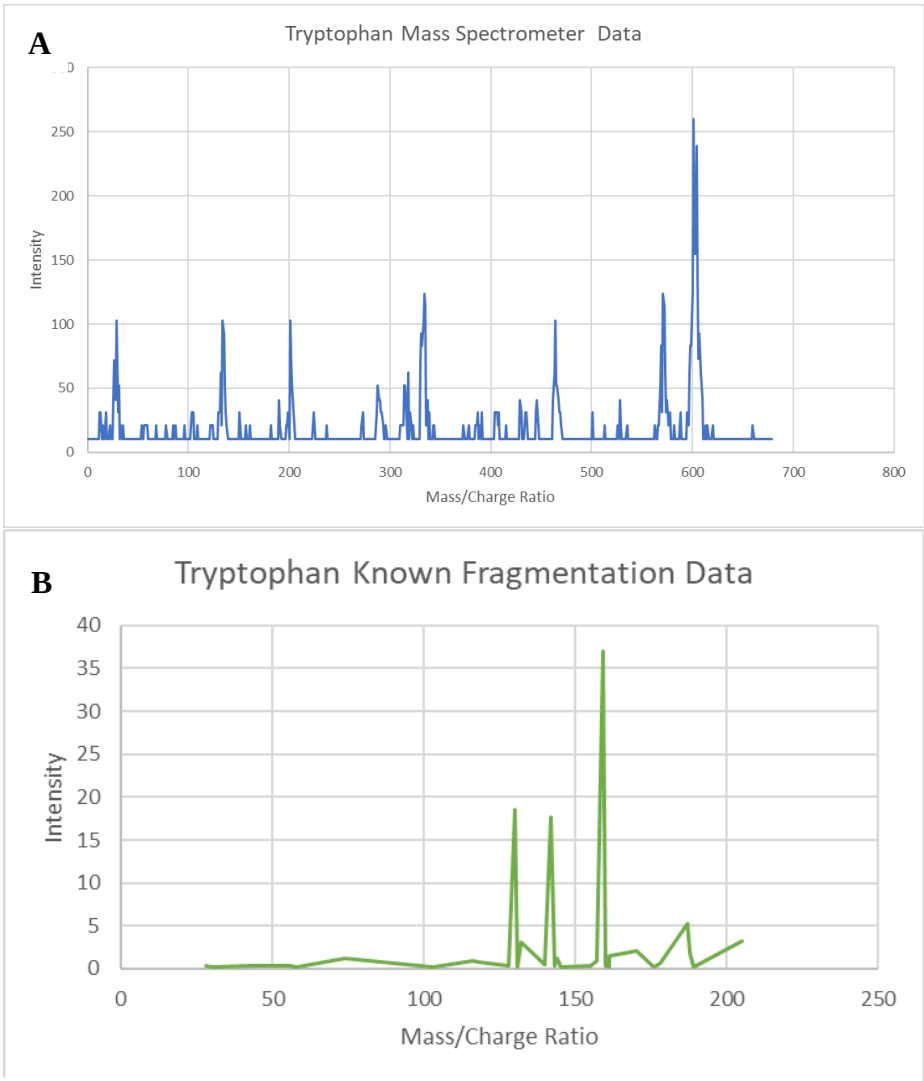

Figure 3:

- **A.** This graph shows raw fragmentation data for tryptophan from mass spectrometer.
- **B.** This graph shows known fragmentation data for tryptophan that was used to confirm metabolite identity[13].

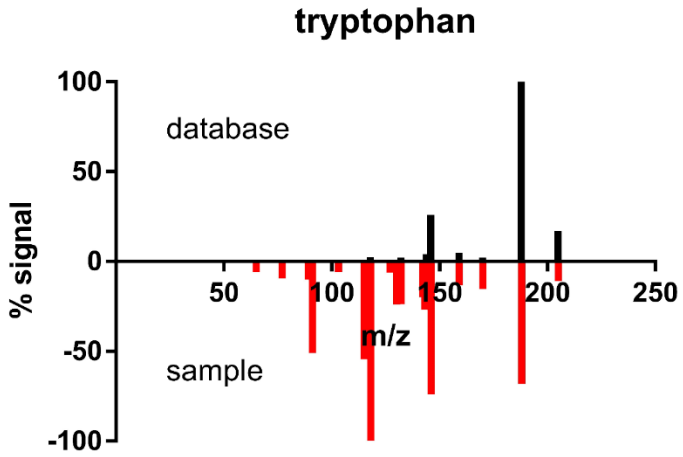

Figure 4: This figure shows the mass fragmentations of the unknown sample and the DataBase fragmentations superimposed for easier comparison.

Statistical tests were conducted to see if metabolites were differentially regulated among the three treatment groups. In order to correctly observe statistical differences, a one-way ANOVA test with Tukey's post-hoc procedure was performed. Once the statistical differences were examined for tryptophan, the same observation and statistical comparison was done on six other metabolites present in the NSC samples (**Figure 5**). For all indicated metabolites, except for alpha-ketoglutarate and serotonin, there were significant differences among the expression levels of the specific metabolite between no IFN-γ group and the soluble IFN-γ or insoluble IFN-γ treatment groups. If there is more than one asterisk presented in the graph, then there is more of a significant difference between the two groups. For instance, the soluble and insoluble IFN-γ treatment groups are significantly different from the no IFN-γ group. However, if one were to examine phenylalanine, they can see that expression levels of phenylalanine from NSCs treated with soluble IFN-γ is much more significantly different from the no IFN-γ group than the expression levels from insoluble IFN-γ treated NSCs compared to the no IFN-γ group (**Figure 5**). There are many changes in amino acids present in NSCs after IFN-γ exposure. This may be due to the potential fact that after IFN-γ exposure, the NSCs perform oxidative phosphorylation. In normal cells, the Citric Acid Cycle generates NADH and H^+ and $FADH_2$ so that they can be used in the Electron Transport Chain to pump H^+ ions into the mitochondrial intermembrane space. Then, the H^+ ions are used during oxidative phosphorylation to generate ATP for the cell. In order to perform these biochemical processes, enzymes have to be used. These different enzymes could be made up of the different amino acids determined in the NSC samples.

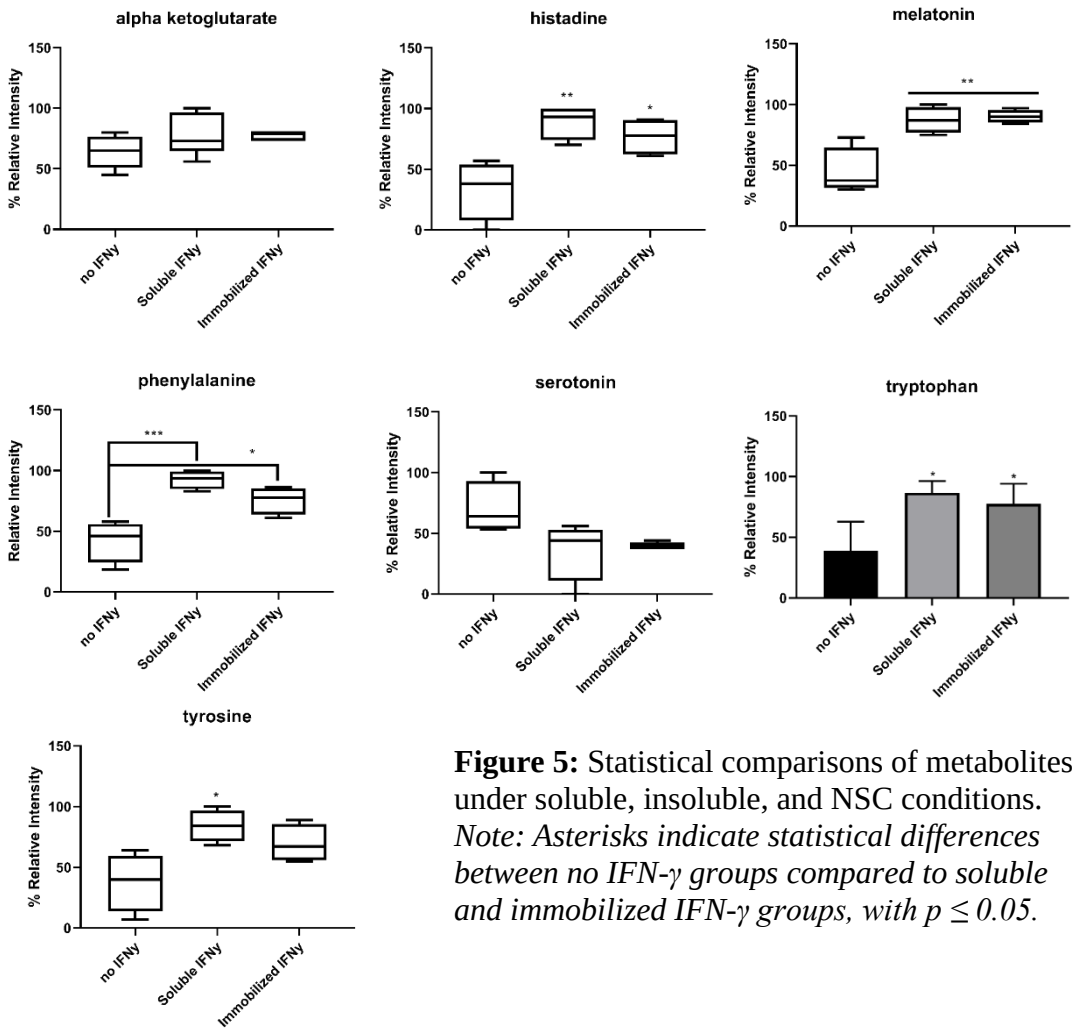

Figure 5: Statistical comparisons of metabolites under soluble, insoluble, and NSC conditions. *Note: Asterisks indicate statistical differences between no IFN-γ groups compared to soluble and immobilized IFN-γ groups, with $p \leq 0.05$.*

Once metabolites were examined for statistical differences, we subsequently used immunohistochemistry to examine the cell organization of NSCs that were seeded into a MAC-DIBO hydrogel. These NSCs were fixed onto a microscope slide, deparaffinized/re-hydrated, and then stained with DAPI so that all cell nuclei could be examined. Once all of the cells were stained appropriately, the microscope slides were examined for the presence of stained cell nuclei (**Figure 6**). In the image, scaffolds are presented as opaque stains on the slides. For instance, **Figure 6A** has its scaffold present in the bottom right corner and **Figure 6B** has its scaffold running down the middle of the slide. The nuclei of the NSCs can be seen scattered throughout the scaffolds. This result indicates that NSCs are present and associated with the MAC hydrogels.

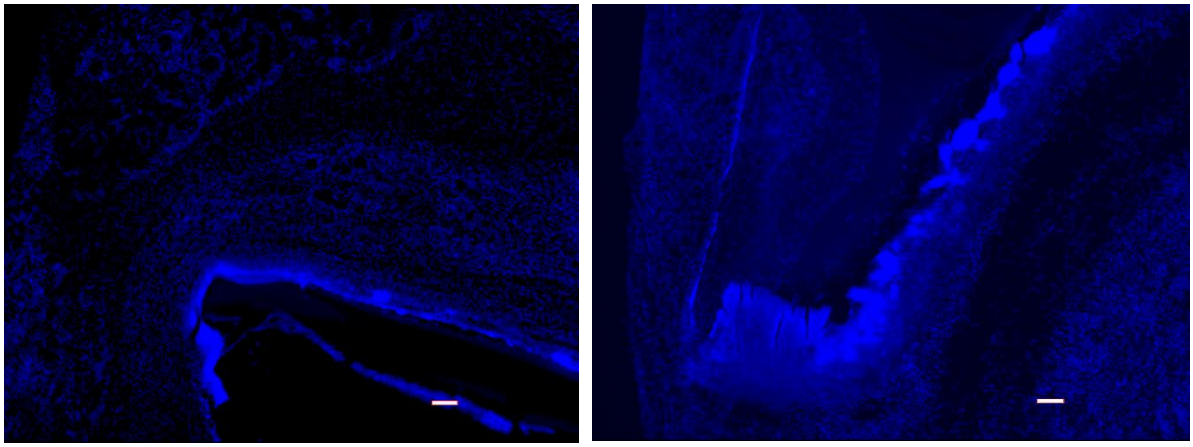

Figure 6: Images of tissue scaffolds (**A** on left and **B** on right) that show embedded and migrating NSCs. *Note:* scale bar indicates 100 µm.

Conclusions:

At the completion of this experiment, our data point to a change in NSCs metabolism, specifically in levels of many aromatic amino acids. This may indicate a switch towards more biosynthesis rather than proliferation. DAPI staining confirmed the viability of the cells within the hydrogel after IFN-γ and future directions will further analyze the identity of these cells.

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Appendix 1: Safety Considerations

While conducting this experiment, many safety considerations had to be kept in mind while working in the lab. While in the lab, general lab safety considerations, such as wearing gloves and goggles, had to be followed. Since this experiment involved using chemicals and stains, gloves had to be worn at all times to avoid irritating the skin and goggles had to be worn to avoid any chemicals splashing into the eyes.

While performing the deparaffinization/re-hydration method, two potentially harmful chemicals were used: ethanol and xylene. Ethanol and xylene are very explosive, flammable chemicals, so they had to be kept away from fire at all times. Not only were those chemicals kept away from fire, but direct inhalation of both chemicals was avoided because they are an internal health hazard. If either of the chemicals were spilled, a paper towel was used to soak up the chemical and wipe off any items that came into contact with the spilled chemical. Then, the paper towel was disposed of into the appropriate chemical waste bin and gloves were changed.

While fixing the cells onto the microscope slides, care had to be taken so that the samples were not contaminated and the microscope slides were not broken. In order to make sure that the cells were not contaminated, gloves had to be worn at all times. Not only were gloves worn at all time, but they were also kept dry to avoid allowing the microscope slide to slip and break onto a hard surface. If a microscope slide was broken, then a hand-held broom and dust pan was used to scoop up the glass and it was deposited into the glass waste bin. After the samples were used, then the slides were disposed of into the biohazard waste containers. Care also had to be taken when handling paraformaldehyde because this chemical is carcinogenic. In order to properly handle this chemical, gloves were worn and all work with this chemical was performed under a chemical hood.

While staining the NSCs with the DAPI stain, care had to be taken because it can stain human skin. Gloves and goggles were worn in order to protect the eyes and skin and care was taken not to touch any surfaces while working with DAPI. If the stain where to get into the eyes or onto the skin, then thorough washing would have taken place on the contaminated area.