SPECIFIC AIM 1. To determine the timing of PAR-1 activation

Original plan: Initially I proposed to use SDS-PAGE/immunoblotting and a recovery assay to determine the timing of PAR-1 activation. Both experiments proved to be problematic. As I mentioned in the submission, the difference in the 45 KDa (unbound PAR-1) and 47 KDa (bound/activated PAR-1) could be difficult to discern and indeed they were. The recovery assay produced a tremendous amount of background that varied from one experimental day to the next, regardless of the different washes we employed. Therefore, we chose to explore the use of RT-PCR.

Findings: Previous studies have shown that PAR-1 activation leads to neuronal apoptosis which is the result of Bid activation. Using this information, we established chick motor neuron cultures, treated with SFLLRNPF (the PAR-1 activator) and collected RNA from these cells at 6 hour time points from 6-96 hours. Although we had to check several primers for both Bid and β-actin (reporter gene), PAR-1 is activated within the first 12 hours of treatment and remains present until 24 hours.

Future steps: Although we had results with the RT-PCR, they are tentative and need to be replicated. Using shared equipment has its advantages and disadvantages. One of the disadvantages with RT-PCR is choosing the setting for the thermocycler. Although we had set up our conditions, we did not discover until the end of the run that the settings had been changed. This resulted in decreased product. Our next step is to repeat this experiment under the optimal conditions, which may allow us to find even a narrow range of PAR-1 activation.

SPECIFIC AIM 2. To demonstrate that RT-PCR is an effective method to determine the subsequent release of second messengers following PAR-1 activation

Original plan: Using the PAR-1 activation information from specific aim #1, we planned to examine changes in 2nd messengers. Due to the difficulty of capturing the exact moment in time when 2nd messenger flux occurs, we proposed to use a downstream indicator of their presence. To demonstrate that RT-PCR is a useful tool in this scenario, we will begin with the hypothesis that increased Ca^{2+} following PAR-1 activation comes from the smooth endoplasmic reticulum (SER).

Findings: Having found that PAR-1 activation results in Bid upregulation between 12 and 24 hours, we used this time point to investigate whether release of Ca^{2+} from the SER was the 2nd messenger in our pathway. Flupirilene, the intended Ca^{2+}, was backordered so we used a treatment of 100 µM ryanodine instead. This concentration of ryanodine has been shown to permanently block Ca^{2+} channels on the SER (Houenou et al., 2004) within 30 minutes of application. Therefore, we treated motor neurons with 100 µM ryanodine 1 hour before adding SFLLRNPF. Our hypothesis was that if PAR-1 activation causes the upregulation of Bid due to the release of Ca^{2+} from the SER, then blocking its release should not result in the upregulation of Bid. RNA isolated from motor neurons

Motor neurons will be collected and cultured as previously described. Since apoptosis of motor neuron cultures is observed at 24 hours, RNA collection will occur at 6, 12, 18, and 24 hours after treatments.
Treatments: Motor neurons will receive one of four treatments: SFLLRNP (to activate PAR-1), thapsigargin (to open Ca\(^{2+}\) channels on the smooth endoplasmic reticulum), fluspirilene (a Ca\(^{2+}\) channel blocker), or FSLLRNP (inactive peptide).

Expected results: If the increase in intracellular Ca\(^{2+}\) is due to release from the smooth endoplasmic reticulum, we should see the same level of caspase-3 expression in the motor neurons that were treated with SFLLRNP and thapsigargin. Caspase-3 expression should be absent from the fluspirilene and FSLLRNP cultures, as these two treatment present our controls. Despite the problem with catching second messenger changes, this method should provide us with more evidence pertaining to the pathway following PAR-1 activation. If this method proves useful, then similar methods will be used to study the subsequent steps in the pathway.

SPECIFIC AIM 3. To demonstrate that the 3D co-culture technology developed by KIYATEC™ (Pendleton, SC) is necessary to examine interactions between neurons and myelinating cells following PAR-1 activation.

Original plan: Although, we have found that myelinating cells express PAR-1 and are subject to similar fates as the motor neurons; however, PAR-1 activation does not result in a total loss of these cells. Using the innovative 3D co-culture technology, we propose a model for treating individual populations separately, but still allowing for interactions between the two populations. This aspect of the grant has been the most exciting, as we have learned more about how to manipulate the 3D culture apparatus and examine how the cells grow in this set-up.

Findings: The initial steps involved studying how our cells would grown in 3D culture and developing our own protocols.

Step 1: Which scaffold works best for motor neurons and glial cells?
My students tested a variety of well-known scaffolds (and multiple concentrations) including collagen and Hydrogel and they found that collagen works best for neurons and glia.

Step 2: How many cells should be loaded into a collagen-filled chamber?
To avoid wasting the 3D cubes, collagen matrices were formed in 96-well plates and seeded with different concentrations of cells. At 24, 48, and 72 hours the number of cells were determined by an MTT assay. The motor neurons grew well at each concentration, with some loss towards the higher end of the time period. Determining the concentration of the proliferative Schwann cells took some time, since they grew more rapidly in the 3-dimensional set-up than when they are grown in traditional cell culture. This resulted in contact inhibition and the loss of many cells. However, after many trials we determined that 1,000 was the initial number of cells that could be seeded into a collagen cube.

Step 3: At what rate should the cubes be perfused with culture medium?
Ideally, we wanted to create an environment that is as close to the physiological environment as possible. We chose the perfuse the cubes at 0.2ml per minute which most closely mimics the movement of interstitial fluid in the spinal cord.

Step 4: How to ensure that the motor neuron axons from the upper cube will grow into the lower cube where glial cells are waiting?
Motor neuron axons are attracted to nerve growth factor (NGF), so 1 mM NGF was applied to the porous filter separating the upper and lower cubes. This concentration was determined from literature research and our own in-house tests. We placed a motor neuron seed collagen cube next to a collagen cube that had been soaked in different concentrations of NGF and waited 48 hours. Then we processed the NGF treated collagen cube for histology by embedding it in paraffin and microtome sectioning it. Since axons contain F-actin, we used anti-phallodii-rhodamine to label the axons. At a concentration of 1mM of NGF motor neuron axons were found in the NGF treated cubes.
Cell survival assays: Motor neurons and myelinating cells (oligodendrocytes or Schwann cells) will be plated separately in the chambers of the culture system. Each chamber will be separately perfused and treated. Although the sample size will be larger to allow for the testing of different time points and statistical analysis, the design will consist of four co-culture systems, so that each cell population is independently treated with SFLLRNP or culture medium. At 24, 48, and 72 hours, the cells will be released from their individual chambers and recaptured in a traditional 96-well plate. An MTT assay will then be used to quantify cell viability.

Myelin production: Following treatment, as described above, cells will be removed from their chambers. Although it is unlikely that myelinating cells could have migrated into the neuronal chamber to deposit myelin proteins, we will collect and assess proteins from all the chambers. Following protein isolation, quantification, and separation on 4-20% tris-glycine gels, a variety of antibodies will be used in western blot analysis to determine the presence of myelin proteins. The KODAK Gel-Documentation Center (Molecular facilities; Furman University) and ImageJ (NIH software) will allow us to determine the levels of protein expression.

Expected results: We have already seen that treatment of the individual cell populations in question each result in decreased survival. However, we need to know how myelinating cells affect motor neurons in a more realistic scenario. If myelinating cells decrease in number or in their ability to make myelin, we should see a decrease in motor neuron cell viability even when they are not directly treated with SFLLRNP. Since there are a variety of proteins involved in myelination, it is difficult to predict which ones might be affected by PAR-1 activation. Regardless, of the outcome a more thorough understanding of this interaction is critical to developing therapies that intervene after an insult to the vasculature during SCI.

References