This is to certify that the
thesis entitled

Identification of Rat Inferior Mesenteric Ganglion Neurons
Differentially Innervating Splanchnic Veins and Arteries

presented by

Ian Behr

has been accepted towards fulfillment
of the requirements for the

Masters degree in Physiology

David J. Kreulen
Major Professor's Signature

December 18, 2009
Date

Identification of Rat Inferior Mesenteric Ganglion Neurons Differentially Innervating Splanchnic Veins and Arteries

By

Ian Behr

A THESIS

Submitted to
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ABSTRACT

Identification of Rat Inferior Mesenteric Ganglion Neurons Differentially Innervating Splanchnic Veins and Arteries

By

Ian Behr

The splanchnic circulation contains approximately 70% of the total body blood volume, the majority in the splanchnic veins. Splanchnic vascular resistance and capacitance can be differentially regulated by the sympathetic nervous system and in some species this is associated with separate arterial and venous innervations. Using an organotypic culture consisting of the inferior mesenteric ganglion along with the arterial and venous arcades in the mesocolon, we identified neurons in the IMG that innervated mesenteric arteries and/or veins by placing retrograde tracers into the vasculature. When fluorescent beads were placed intravascularly, optimal filling of neuron cell bodies required 7 days of culture and required intact lumbar colonic nerves. If beads were placed in the inferior mesenteric artery adjacent to the IMG, blood vessels within the ganglion filled with tracer identified an extensive intraganglionic vascular network and obscured clear visualization of neurons. Thus in all experiments tracers were excluded from the adjacent inferior mesenteric artery. If beads were injected into the entire mesenteric arcade 65% of identified neurons contained the tracer. When beads were instilled into distal branches of the vasculature only 10% of identified neurons contained beads. Of the 10%, 58% contained beads from veins and 42% contained beads from arteries. These studies suggest that separate populations of neurons in the IMG innervate mesenteric arteries or veins.

Dedicated to my family, my dad Jeff, mom Diana, sisters Alyse and Danielle, and grandparents Lon and Richard Behr, who without the support of I would not be where I am today.

ACKNOWLEDGEMENTS

I would like to thank both the past and present members of the Kreulen lab who have helped and made coming into the lab every day all the easier. I would also like to thank Dr. David Kreulen who was not just a P.I. to talk to, but a friend who I could be around both in and out of the lab. I would also like to thank the histology department for all of the work that they helped me with, putting up with my frequent visits down the hall to see how things were going. Lastly I would like to thank my committee members, Dr. David Kreulen, Dr. Robert Wiseman, and Dr. Laura McCabe for their support in this project.

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Images in this thesis are presented in color

KEY TO ABBREVIATIONS

IMG	**Inferior Mesenteric Ganglion**
BP	**Blood Pressure**
CVD	**Cardiovascular Disease**

SPECIFIC AIMS

PROJECT

Previous research suggests veins may be involved in the control of blood pressure and that neurogenic venoconstriction may contribute to some forms of hypertension (Fink, 2008). Veins contain more than 70% of the body's total blood volume and may be important in the control of blood pressure. Separate neuronal pathways for veins and arteries have been documented in some animal species but this innervation is controversial in a rat model. Neurons have been shown to innervate both veins and arteries separately and together when different retrograde labeling methods were used (Browning *et al.*, 1999;Dehal *et al.*, 1992;Hsieh *et al.*, 2000). To characterize the rat inferior mesenteric ganglion retrograde labeling will be used to determine the pathways for both veins and arteries.

SPECIFIC AIM I: <u>Develop Retrograde Tracing Method</u>

A. Develop a method to inject retrograde tracers into veins and arteries in an intact inferior mesenteric ganglion using an *ex-vivo* organotypic tissue culture preparation.

B. Determine the time course of retrograde filling and the optimum concentration of retrograde tracers.

C. Optimize methods for visualization of retrograde tracers in inferior mesenteric ganglion.

SPECIFIC AIM II: Determine the Number of Neurons in Rat IMG

SPECIFIC AIM III: Determine the proportion of Venous and Arterial Neurons

Introduction

Stroke, myocardial infarction, and heart failure are a few examples of cardiovascular disease (CVD), a pathophysiology which accounts for more than one third of all global deaths (World Health Report 2002). The largest cause of CVD is inadequate control of blood pressure (BP). Arterial blood volume is the main determinant of long-term, steady-state BP (Grisk & Rettig, 2001;Johnson *et al.*, 2002;Lifton *et al.*, 2001). Systemic hemodynamics and BP are regulated by changes in vascular capacitance and resistance primarily in the veins and arteries, respectively. The body's largest capacitance and resistance bed, the splanchnic vasculature, is innervated by the prevertebral sympathetic ganglia, which include the celiac, superior and inferior mesenteric ganglia. Sympathetic nerve-mediated increases in arterial resistance and decreases in venous capacitance can be evoked separately by activating the sympathetic nerves at varying frequencies: the capacitance decrease develops at lower frequencies than the resistance increase (Karim, 1976;Hainsworth, 1983). It is not known whether the separate regulation of arterial resistance and venous capacitance by the sympathetic nervous system resides in a separate innervation of the arteries and veins by specific sympathetic ganglion neurons. This study aims to identify neurons in the inferior mesenteric ganglion (IMG) that innervate splanchnic veins and arteries and to determine whether there are vessel-specific neurons within the IMG.

Splanchnic vascular hemodynamics are primarily regulated through the nervous system. The splanchnic system is innervated by sympathetic nerves

projecting from the inferior mesenteric, superior mesenteric, and celiac ganglia, as well as by sensory nerves projecting from spinal dorsal root ganglia. Up to two thirds of the blood stored in the splanchnic veins can be mobilized by sympathetic nerve stimulation (Greenway, 1983). Sympathetic reflex responses can contribute more than 30% of the body's increase in blood pressure during hemorrhage (Lundgren, 1983). Elevated blood pressure and hypertension have been correlated to increased sympathetic activity (Reid, 1975;Anderson, 1989;Schlaich, 2004) While the documentation of vessel specific neuronal pathways has been controversial, venous specific prevertebral innervation could indicate that perhaps veins physiologically react differently than arteries during the onset of hypertension.

Different sympathetic control mechanisms specific for veins or arteries have been identified. Stimulating nerves that innervate arteries elicit rapid excitatory junction potentials. No such excitatory junction potentials are recorded from veins when sympathetic nerves are stimulated (Kreulen, 1986;Suzuki, 1981). When veins and arteries are exposed to repetitive stimulation at the same frequency, veins proportionally more than arteries. (Kreulen, 1989) Arterial excitatory junction potentials result from the release of ATP from sympathetic nerves acting on P2X ligand-gated receptors of vascular smooth muscle cells (Evans, 1992). Venous smooth muscle cells contain P2Y G-protein coupled receptors that don't elicit excitatory junction potentials in the presence of ATP (Galligan, 2001).

. Distinct populations of arterial and venous sympathetic neurons were indentified in guinea pig IMG when retrograde tracers were placed intravascularly in the inferior mesenteric artery and vein *in vitro* (Browning, 1999). Similar separate populations of arterial and venous neurons were found in paravertebral sympathetic ganglia that innervate the femoral artery and vein (Dehal, 1992). In contrast, both distinct arterial and venous neurons as well as neurons that innervated both arteries and veins were found in the rat celiac ganglia when retrograde tracers were placed extravascularly around mesenteric veins and arteries *in vivo* (Hsieh, 2000). The purpose of this study is to determine the pattern of innervation of mesenteric veins and arteries by neurons in the rat IMG.

SPECIFIC AIM I Experimental Method Development

AIM I A. INTRODUCTION

The Inferior Mesenteric Ganglion has previously been studied by Kreulen et al in both guinea pig and rat (Kreulen, 1982;Kreulen, 1986). However, retrograde labeling studies in an *ex-vivo* tissue culture preparation have only been performed in guinea pigs. Hsieh et al performed similar retrograde labeling in rats, however their technique used retrograde tracers applied on the outside of arteries and veins, areas susceptible to possible non-specific interactions between venous and arterial axons (Hsieh *et al.*, 2000). This section of experiments will detail how venous and arterial blood vessels are separately injected into and imaged with confocal microscopy for analysis.

AIM I A. Methods

Ex-Vivo Dissection and Injection Technique

Male rats (100-114g) were euthanized with pentobarbital (Fatal Plus®, 0.05mg/kg, Vortech Pharmaceuticals/Dearborn Michigan) and exsanguinated. The abdomen was opened and the small intestine and part of the large intestine was pushed to the side in order to expose the colon. The gonads were removed in order to better visualize the IMG and surrounding structures. The pubic bone was then cut rostral-caudally, and spread apart in order to expose the colon covered beneath. The colon and all tissue deep to the pubic symphesis were then cut down to the spine with a ventral to dorsal incision. The colon was then lifted at the incision point and the all the tissue between it and the spine was cut moving up to the kidneys while continuing to cut rostral/caudally against the spinal column.

The colon and attached tissue was then removed from the animal. Once the tissue was cut up to the kidneys, the removed tissue was placed into a petri dish for further dissection under a dissecting microscope. Hanks salt solution warmed to 37° C. The preparation was pinned out using the two ends of the colon and the two ends of the aorta. Muscle, fat, the spermatic artery, and other tissues surrounding the aorta were cut.

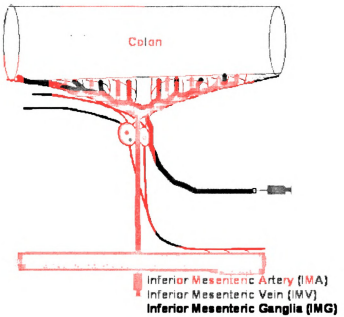

Figure 1. Schematic Representation of Rat Inferior Mesenteric Ganglion Preparation.

To inject into the artery, the aorta was cut open opposite to the iliac artery branch point. Once the opening to the inferior mesenteric artery was located, a 30 gauge needle was inserted into the hole and liquids were injected through the blood vessels. Without cutting/opening other blood vessels, injecting through the inferior mesenteric artery flushed liquid all the way through the venous blood vessels along with the arterial vessels. However, in order to get liquid to flow through and not just build up pressure in the vessels, openings of the blood vessels adjacent to the ends of the colon. De-ionized water and phenol flushed through the blood vessels were used to wash away endothelial cells to enhance uptake of fluorescent retrograde tracing beads or chemicals. All of the blood vessels were flushed first with de-ionized water, followed by 25% phenol, hanks salt solution, and then retrograde tracers were injected.

Tracers were injected into the inferior mesenteric vein proximal to the colon near the coloninc branches of the inferior mesenteric artery. The mesentery and artery around this area was cut away so that just the v shape of the bifurcating vein was isolated. In the middle of this V a small cut was made. Through this cut the top half of a pair of iridectomy scissors was placed to fillet open the vein. This opening was then large enough to accommodate an injection needle. This same procedure was also done on the artery, however it was difficult and did not work often. The inferior mesenteric artery was small, less compliant, and easier to tear.

Once openings in the blood vessels were made, the mesentery was pinned out above the blood vessels between the colon. Veins and arteries

between the colon and IMG were cauterized prior to removal of the colon from the preparations. Cauterization of these blood vessels severed the connection between arterial and venous blood vessels, thus preventing crossover of tracer from vein to artery. One color fluorochrome was injected into arterial blood vessels, and another into venous blood vessels.

Confocal Microscopy

After 7 days incubation at 37° C, preparations were fixed for 2 min in 4% paraformaldehyde. Fixed preparations were placed on a glass slide and examined. Using an Olympus Fluoview™ Confocal Microscope located in the Center for Advanced Microscopy at Michigan State University, images were taken of *ex-vivo* tissue preparations. Z-series dimensional imaging was used to capture the entire preparation. 5µm optical slices were made through the entire thickness of the preparation, stacking the slices together into one image.

AIM I A. Results

Confocal microscopy identified successful injection of retrograde tracing beads into both veins and arteries. Overlap between artery and vein specific fluorochromes was not observed when looking at the whole tissue preparation (Figure 2 A-D), and when focused on top of the IMG (Figure 2 E-H).

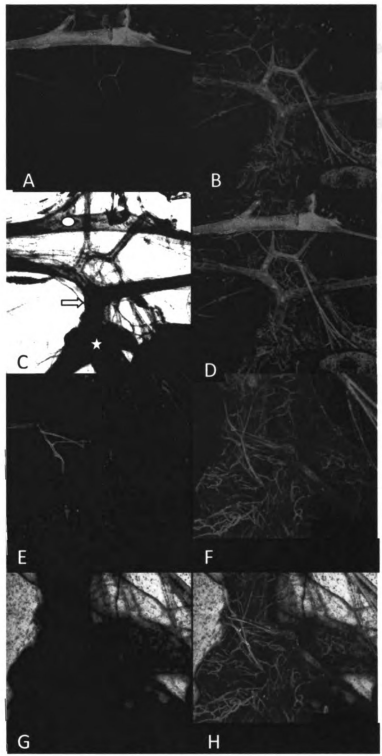

Figure 2. Confocal Microscopy of Ex-Vivo Preparation Injected With Red Fluorescent Beads in the Arteries and Green Fluorescent Beads in the Veins. Images A-D 4x, E-H 20x, (A) Green Channel Only, (B) Red Channel Only, (C) Transmitted Light, arrow pointing to artery bifurcation, star over ganglia, circle over vein, (D) Red-Green Overlay. (E) Green Channel Only, (F) Red Channel Only, (G) Transmitted Light, (H) Red-Green Overlay.

AIM I B. Introduction

The next objective was to confirm that retrograde tracers were working, and that neurons could be successfully labeled. In order to do this a pre-ganglionic severed nerve fiber running to the inferior mesenteric ganglion was bathed in retrograde tracers instead of inside a blood vessel.

AIM I B. Methods

Ex-vivo tissue preparations were dissected as previously explained. The splanchnic nerve was then cut. The severed nerve was placed in a hole molded out of the agar of a dissection petri dish. Retrograde tracers were then put into the well with the cut nerve. Vaseline was used to cover the nerve and fluorochrome in order to seal the tracer around the nerve so a high concentration of fluorochrome remained around the fiber only. The nerve/tissue was incubated for three days and then the ganglia were imaged using confocal microscopy as described above.

AIM I B. Results

Fluorescently labeled neurons were visualized when nerve fibers were bathed with either green 0.05 µm green-yellow beads purchased from Molecular Probes, red 0.05 micron red beads purchased from Duke Fluorescence, or Fast Blue purchased from Ambion, filled neurons most successfully. Neurons retrogradely labeled with fluorescent beads appeared to have granular fluorescence filling cells up, whereas those labeled with Fast Blue had their entire cytoplasm filled with solid non-granular fluorescence (Figure 3). The cluster of neurons filled with tracers was closest in proximity to where the bathed nerve fiber entered the body of the ganglia. Distal to the bathed fiber there were no cells that could be identified as successfully taking up retrograde tracers. Fast Blue also filled smaller non-neuronal cells within the ganglia in addition to neurons.

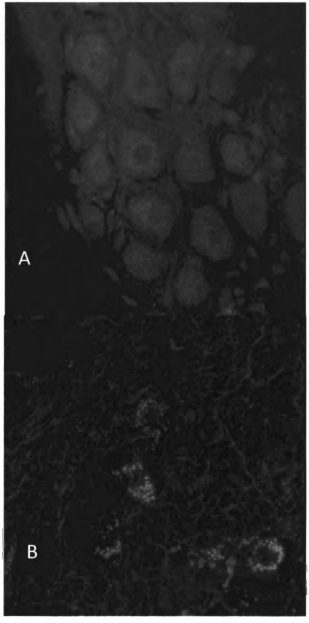

Figure 3. Neurons labeled with retrograde tracers from severed nerve bathed in tracer for three days. A-B 40x A. Fast Blue labeled cells B. Green Bead labeled cells.

AIM I C. Introduction

From the results above, we found neurons were most successfully labeled with the glass bead fluorochromes and the chemical fluorochrome Fast Blue. We therefore continued with the use of these retrograde tracers to determine how well we could label neurons through blood vessels filled with these fluorochromes. Studies of the vascularization of other ganglia, such as the DRG (Jimenez-Andrade *et al.*, 2008), have shown that there is a large concentration of blood vessels encompassing some ganglia. The presence of the vasculature of the IMG has not been previously documented.

Specific Aim I C will determine whether fluorescent tracers are filling nerve fibers and/or blood vessels surrounding the inferior mesenteric ganglia. We will also determine the optimal incubation duration necessary to fill the maximum number of neurons. Control experiments were performed to evaluate the specificity of labeling to ensure that the neurons were only taking up tracer from within the vessels and not from the medium.

AIM I C. Methods

Fluorochrome Concentration Determination

Various dilutions of retrograde tracing beads were used for 3 day incubations of ex-vivo preparations to determine what concentrations worked best. These concentrations ranged from a 1:10 dilution through a 1:10,000 dilution. The same retrograde tracer was injected into both the veins and the arteries through the opening of the inferior mesenteric artery from the aorta. Fast Blue was then applied directly to the ganglion in order to observe the quantity of cells present filled with retrograde tracers.

Identification of Blood Vessel Network Surrounding Inferior Mesenteric Ganglia

Inferior Mesenteric Ganglion dissections were performed as previously described. Both whole *ex-vivo* preparations as well as isolated ganglia were fixed for 24 hours in ZINC FIX from BD Biosciences. Tissue was then rinsed in PBS and placed into blocking solution for 60 minutes at room temperature. CD31, platelet endothelial cell adhesion molecule, primary anti-body was then bathed around tissue in a 1:500 dilution overnight at 4 degrees Celsius. Samples were then rinsed in two changes of TBS+TW20 for 5 minutes each rinse at room temperature. Secondary DyLight549 Anti-body was then incubated with the sample at a 1:600 dilution for 3 hours at room temperature. Samples were then washed with 3 changes of TBS for 10 minutes and mounted on slides.

Control Experiments

Control experiments were ran placing retro-grade tracing beads on top of and around the IMG for 3 days. Fast Blue was then applied to the ganglion to identify neurons within the IMG and determine if they had taken up fluorescent beads. In addition to this control, tissue preparations were also imaged after the post ganglionic nerve fibers were cut. In order to do this, a rostral to caudal incision was made underneath primary blood vessels of the inferior mesenteric artery.

AIM I C. Results

Fluorochrome Concentration Determination

Duke Fluorescence Red Tracers were best visualized in a 1:100 dilution, Molecular Probes yellow-green bead tracers in a 1:1000 dilution, and Fast Blue worked best at a 1% concentration. These concentrations allowed optimal detection with minimal noise. However, there appeared to be what we termed the meshwork effect around the ganglia during imaging after all experiments. A meshwork of wire looking vessels encompassing the ganglia, either blood vessels and/or axons, appeared to be full of fluorescence. This made it difficult to say whether cells, blood vessels, or axons were fluorescing in certain areas. It was determined that approximately 65% of cells labeled were positive for retrograde labeling when both the arteries and veins were filled with a single tracer (Figure 4 and 5).

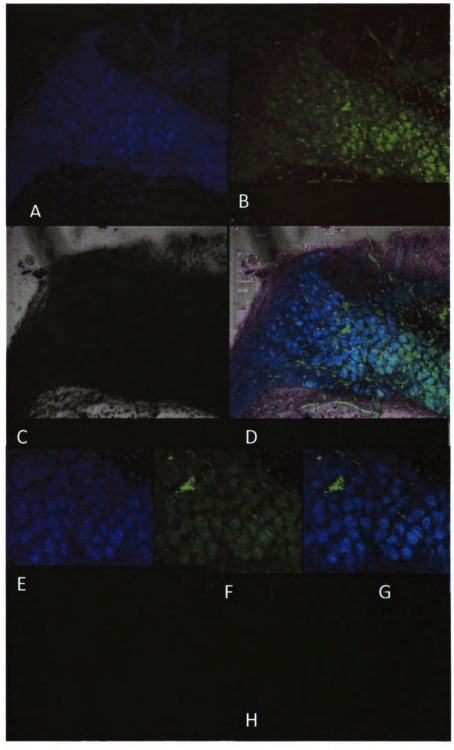

Figure 4. Neurons Filled with Green Beads and Instilled with Fast Blue. Images A-D 10x, E-H 40x (A) Blue Channel, (B) Green Channel, (C) Transmitted Light, (D) All Channels Merged, (E) Blue Channel, (F) Green Channel, (G) Blue Green Merge, (H) Transmitted Light.

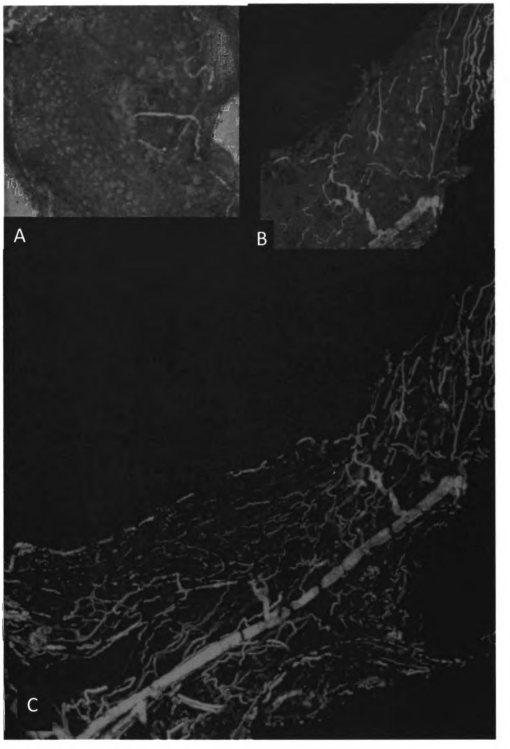

Figure 5. Neurons Filled with Red Beads and Instilled with Fast Blue. Image A-B 10x, C 4x.

Identifying Inferior Mesenteric Ganglion Vascularization

Through histological techniques, we were able to identify the "Chicken Wire" as blood vessels encompassing the tissue (Figure 6). The IMG was highly vascularized.

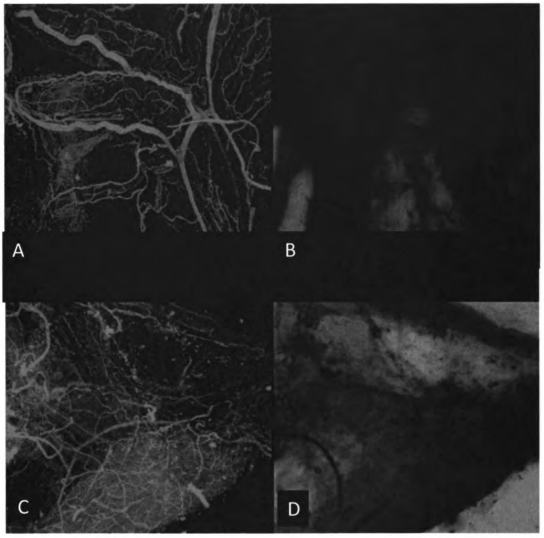

Figure 6. CD31 Histological Staining of Inferior Mesenteric Ganglion Blood Vessels. A/B 4x, ganglia attached to the inferior mesenteric artery and mesenteric tissue surrounding,(A) Red channel, (B) Transmitted Light. C/ D 20x, ganglia removed from inferior mesenteric artery, (C) Red Channel, (D) Transmitted light.

Control Experiments

When Fluorescent Retrograde tracing beads were placed on top of the tissue, no neurons that picked up tracers could be identified. Small patches of fluorescence were observed around the edges of the tissue, most likely a result of fluorescent beads being caught in the mesentery (Figure 7).

When the mesentery and post ganglionic fibers were cut, no fluorescent cells were observed (Figure 8). This demonstrates that our cells previously observed were a result of retro grade filling from blood vessels, not a result of retrograde filling from tracers leaking into the culture medium.

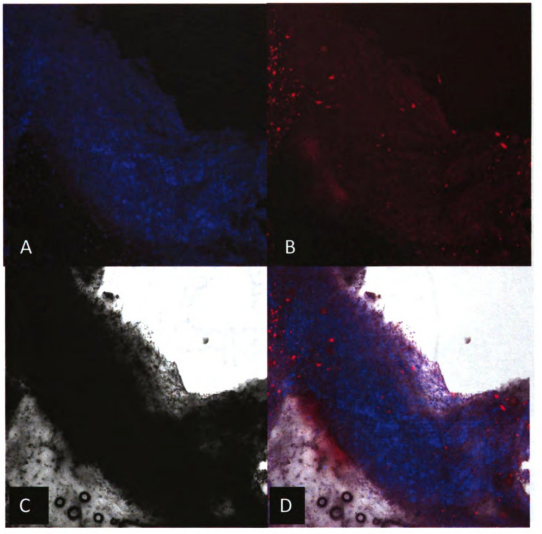

Figure 7. Control Tissue with Retrograde Tracer Application on Top of Sample and Fast Blue Instilment. Images A-D 10x, (A) Fast Blue Channel, (B) Red Bead Channel, (C) Transmitted Light, (D) Merge of all three channels.

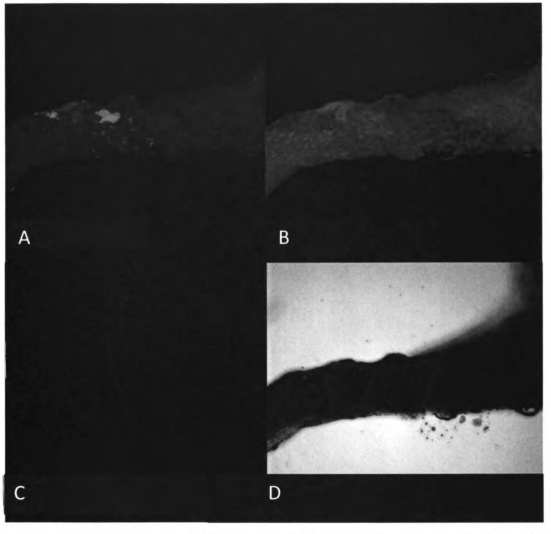

Figure 8. Severed Nerve Fiber Control Sample. Images A-D 10x, (A) Red Filter Only, (B) Blue Filter only, (C) Green Filter Only, (D) Transmitted Light.

AIM I D. Introduction: Whole Mount Ganglia Dual Fluorochrome Injections

The results above demonstrated that neurons positive for retrograde labeling appear dimmer than the blood vessels full of fluorescent retrograde tracers encompassing the ganglia. As a result it was difficult to observe all of the cells that might innervate veins and/or arteries. In order to distinguish neurons from blood vessels, after tracer injections and incubation of the tissue, phenol was injected into the inferior mesenteric artery in an effort the flush the fluorescent beads out of the IMG blood vessels. This section of aim I uses two retrograde fluorochromes injected separately into veins and arteries to determine whether inferior mesenteric neurons differentially innervate veins and arteries.

AIM I D. Methods: Whole Mount Ganglia Dual fluorochrome Injections

Tissue samples were dissected and injected into with retrograde tracers as described above. In addition to the previous stated methodology, 25% phenol was injected in the mesenteric artery after the tissue was incubated. This was followed by two washes with hanks salt solution. Samples were then fixed and mounted on slides in a similar fashion as previously explained.

AIM I D. Results: Whole Mount Ganglia Dual fluorochrome Injections

After injecting, incubating, and flushing tissue samples out with phenol, confocal imaging revealed fluorochrome filled neurons where 99% were positive for both venous and arterial innervation. All retrograde tracers demonstrated similar results (Figures 10 and 11). While the use of Fast Blue resulted in a more thorough labeling of neurons compared to beads, this chemical also labeled non-neuronal cells

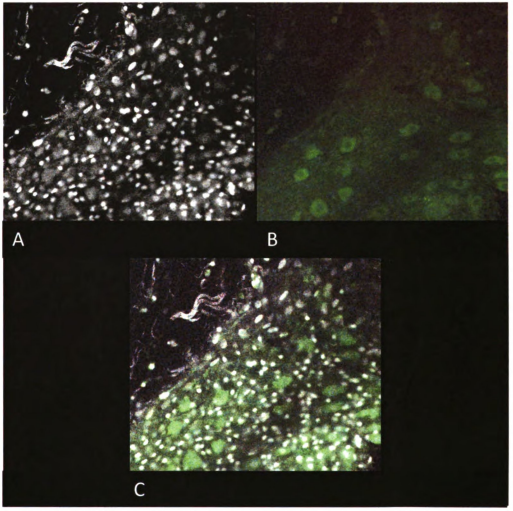

Figure 9. Dual Injected IMG Preparation with Fast Blue in Vein and Green Beads in Artery. Images A-C 40x, (A) Blue Channel, (B) Green Channel, (C) Blue Green Merged Image.

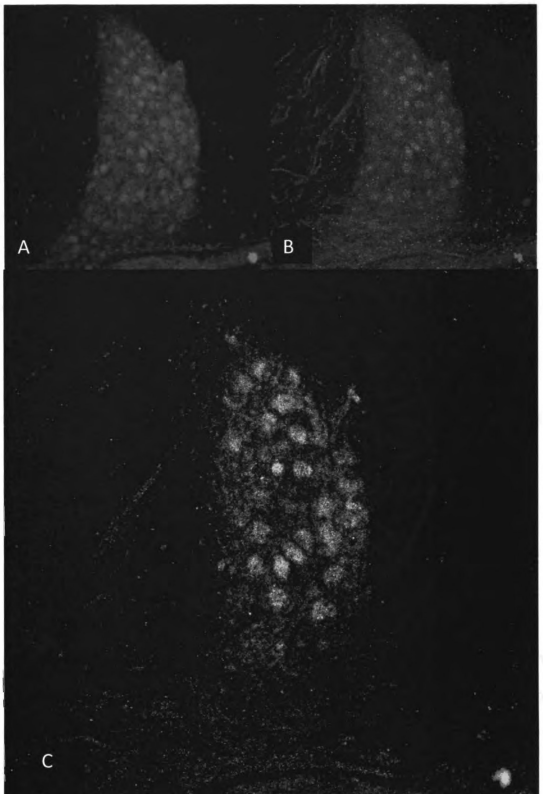

Figure 10. Dual Injected IMG Preparation with Green Beads in Artery and Red Beads in Vein. Images A-C 40x, (A) Red Chanel, (B) Green Channel, (C) Red Green Merged Image.

AIM I E. Introduction: Determine how to best visualize cells and avoid blood vessels encompassing the ganglia

Cyrostat Sectioned Ganglia

Cyrostat sectioned IMG preparations were used to better observe neurons. Sectioned tissue was used to decrease the fluorescence from the blood vessels encompassing the IMG. In addition neurons which would normally be on top of each other in un-sectioned ganglia are easier to observe in separate sections of IMG.

Spectral Imaging

Spectral Imaging is a Confocal Microscopy technique whereby fluorescence can by precisely determined to be specific to a fluorochrome of interest. Largely accomplished through computer software, this technique directly shows whether neurons are positive for venous and or arterial fluorochrome filling.

Blood Vessel Distal Injection Protocol

All of the blood vessels to this point have been filled with retrograde tracers by injecting arterial fluorochromes through the opening of the inferior mesenteric artery from the aorta. The venous circulation was injected into from a position adjacent to the colon. These injection points result in filling of the blood vessels encompassing the IMG identified in specific AIM I, making it difficult to identify labeled cells. The goal of this portion of specific AIM II is to determine how to inject retrograde tracers into a distal segment of inferior mesenteric artery

and vein in order to avoid the filling of the vasculature surrounding the inferior mesenteric ganglion.

AIM I E. Methods: Determine how to best visualize cells and avoid blood vessels encompassing the ganglia

Cyrostat Sectioned Ganglia

Tissue samples were collected and tracer injections were performed as previously explained. After fixation, the IMG and any of the adjacent inferior mesenteric artery it surrounded was removed from the rest of the tissue. Samples were then put into a 30% sucrose solution until the tissue became buoyant in the liquid, approximately 20 minutes of bathing time. Samples were given to the HistoPathology Lab located on the second floor of the biomedical physical sciences building at Michigan State University. Samples were frozen and cut using a cryostat into 10*u*m thick sections. After mounting onto slides these samples were stained with toluidine blue in order to better visualize neurons.

Spectral Imaging

The Olympus Fluoview Confocal Microscope located in Michigan State's Center for Advanced Microscopy was used for spectral unmixing analysis. A sample IMG of dually injected fluorochromes was first imaged. 10nm step intervals of emitted wavelengths of light were recorded, ranging from 400nm to 800nm. Samples of the fluorescent tracers being used were separately put onto microscope slides and imaged in a similar fashion. Lastly, an IMG sample fixed and sectioned in the same manner as all other IMG preparations, but not injected into with retrograde tracers was tested. Imaged again in the same way, this sample would reveal if any auto-fluorescence light emission was occurring.

Combined together, these spectrums of light were put into the Olympus computer software where pixel by pixel reconstruction of wavelengths of light not specific to the spectrum of the fluorochromes of interest was subtracted from a final image of the dually injected IMG.

Blood Vessel Distal Injection Protocol

Instead of injecting into the whole inferior mesenteric vein, one quarter of the blood vessel was separated by cauterization. In order to inject into the mesenteric artery, all the surrounding tissue had to be cleared away so that a v shaped bifurcation similar to the vein was isolated adjacent to the colon. A segment of mesenteric artery similar in length to the isolated vein was separated by cauterization. Using forceps, the tissue was pinched in the middle of the v so that a small piece of tissue was pushed above the forceps. With a pair of iridectomy scissors, a cut was made creating a small hole in the top of the mesenteric artery. The injection needle was then placed against this opening and tracer was forced into the desired segment of inferior mesenteric artery.

AIM I E. Result: Determine how to best visualize cells and avoid blood vessels encompassing the ganglia

Cryostat Sectioned Spectrally Unmixed Ganglia

Cyrostat sectioned tissue samples revealed the same results as whole mount ganglia when dually labeled IMGs were imaged with Spectral Unmixing; all cells labeled appeared to be positive for both arterial and venous innervation (Figures 12/13). Autofluorescence was detected, however through the computer software, this light emission was able to be subtracted from the rest of the sample. Venous and arterial fluorescence was determined to be specific to our retrograde tracers used.

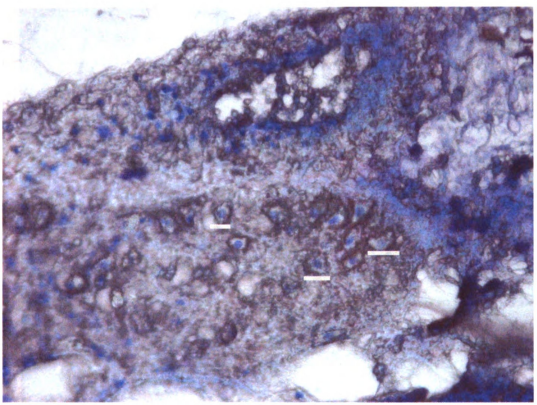

Figure 11. Brightfield Image of 10μm Cryostat Sectioned Inferior Mesenteric Ganglion Stained with Toluidine Blue. Obtained through confocal imaging with a 20x objective. Neurons are underlined.

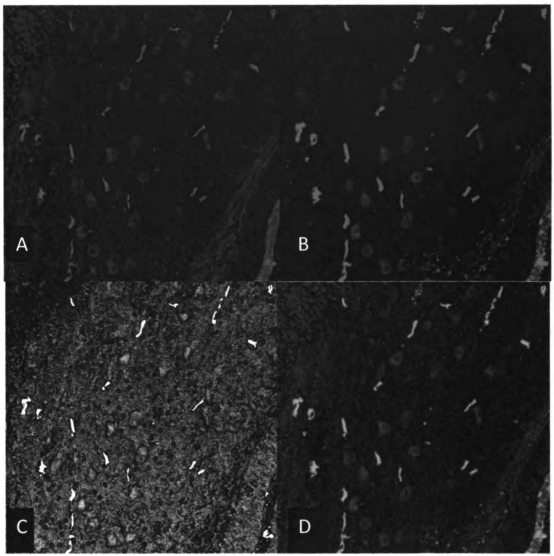

Figure 12. Cryostat Sectioned IMG with Green fluorochrome in Veins and Red fluorochrome in Arteries. Images A-D 20x, (A) Green Channel Only, (B) Red Channel Only, (C) Auto-fluorescence, (D) Green-Red Merge with auto-fluorescence subtracted/unmixed from image.

SPECIFC AIM II Neuron Demographic Characterization

AIM II. Introduction

A whole mount inferior mesenteric ganglia tissue preparation is approximately 150-250 *u*m in thickness and cannot be analyzed histologically. Furthermore, there is a considerable amount of connective tissue that encompasses the neurons of interest in the IMG. As a result all of the cells within the tissue cannot be marked with antibodies.

It was therefore important to find another method of labeling that could be used to quantify the number of neurons in a rat IMG. Previous experiments have been performed in rats to label peripheral nerves through retrograde transport using the fluorochrome Fast Blue (Puigdellivol-Sanchez *et al.*, 2000). This fluorochrome is also capable of labeling cells if applied on top of the tissue. Fast Blue was applied directly on top of an inferior mesenteric ganglion tissue preparation in order to efficiently identify the number of cells in an IMG.

AIM II Methods

Fast Blue is most efficient when used between a concentration of two and five percent (Puigdellivol-Sanchez *et al.*, 1998). However, the use of Fast Blue injected into blood vessels in large volumes has not been documented. For these experiments a solution concentration of 0.5% was used.

Neurons that have filled with the fluorochrome after one day of incubation were visualized with confocal microscopy. We also applied fast blue 4, 5, and 6 days after tracer injection, imaging on the 7^{th} day. It was difficult to get Fast Blue to stay concentrated around the ganglia. Cutting a small hole in the mesentery that surrounds the ganglia, filling the mesentery like a balloon with tracer localized directly around the ganglia yielded the most success.

AIM II Results

Approximately 150±26 (n=5) inferior mesenteric neurons were labeled per IMG tissue preparation, (Figure 9). Labeled neurons were located at the center of the ganglion. The longer the tissue preparation was incubated out of the animal, when Fast Blue was instilled fewer cells were labeled.

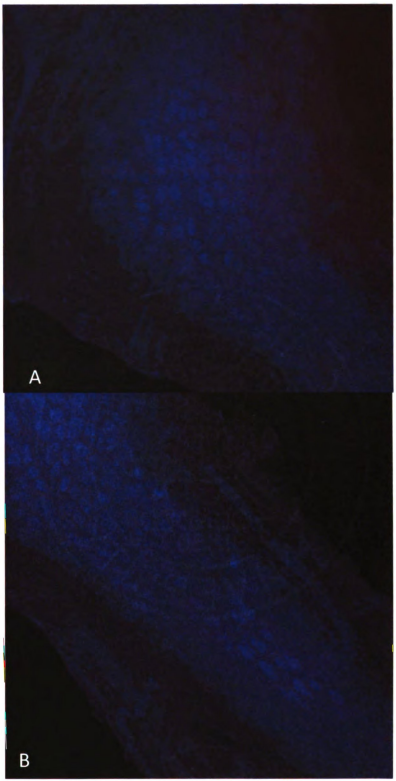

Figure 13. Inferior Mesenteric Ganglion Neurons Labeled With Fast Blue. (A) top of ganglion, (B) bottom of ganglion.

SPECIFIC AIM III Introduction. Blood Vessel Distal Injection Combined With Cryostat Sectioning and Spectral Unmixing

From the results in specific aim I above, in order to best visualize retrograde filled neurons in the inferior mesenteric ganglia, a combination of techniques needed to be applied. To avoid the vasculature encompassing the ganglia, blood vessels distal to the IMG were filled with fluorochromes. After incubating tissue preparations, samples were then sectioned to better visualize neurons. Spectral unmixing analysis was used to differentiate fluorochrome specific fluorescence. These techniques combined together will determine if neurons of the IMG are retrograde labeled and innervate splanchnic vasculature.

AIM III Methods.

All of the methods used in this section are described above in section I.

AIM III Results. Blood Vessel Distal Injection Combined With Cryostat Sectioning and Spectral Unmixing

When IMG samples were dually injected into distal venous and arterial blood vessels, cells positive for only venous or only arterial innervation were observed; see Figure 14 below. Not as many cells were found to be positive for fluorescence, but this was expected as a result of only a portion of the blood vessels in the tissue preparation being filled with fluorescent retro-grade tracers.

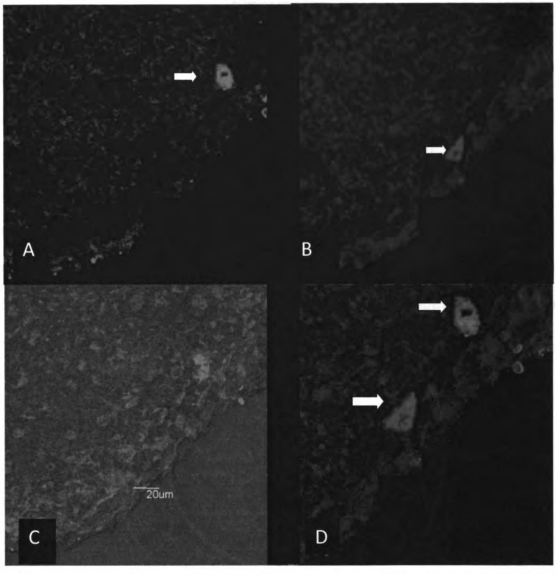

Figure 14. Cryostat Sectioned Spectrally Unmixed IMG Sample Injected with Fast Blue in the Veins and Red Beads in the Arteries. Images A-D 20x, (A) Red Channel Only, (B) Blue Channel Only, (C) Auto-fluorescence channel, (D) Red-Blue Merge with auto-fluorescence subtracted/unmixed from image.

DISCUSSION

This study confirms the existence of two separate populations of inferior mesenteric ganglia neurons, one innervating mesenteric veins, and another innervating mesenteric arteries. These results are consistent with the findings of both Browning and Dehal et al (Browning *et al.*, 1999;Dehal *et al.*, 1992). Only a sub-population of IMG neurons were positive for single innervation of venous and arterial vessels. It is also argued that there are neurons which innervate veins and arteries together when retrograde tracers are bathed exrtavascularly(Hsieh *et al.*, 2000). Our results showed when blood vessels encompassing the ganglia were filled with retrograde tracers, cells positive for both venous and arterial innervation were labeled.

The retrograde tracing methodologies described herein provided previously unreported information about basic characteristics of the IMG. Fluorescent marking of neurons with Fast Blue showed a population of ~150 neurons within the IMG of a male rat. From this population, 65% were retrogradely labeled by injecting a single retrograde tracer into both the venous and arterial circulation of the entire preparation.

Wire like fluorescence observed in these experiments was identified to be a network of blood vessels encompassing the IMG. Similar techniques identified the same pattern of vascularization in dorsal root ganglia (Jimenez-Andrade *et al.*, 2008). These results suggest all sympathetic ganglions may be highly vascularized. The vascular supply of the IMG which we identified has not been previously described. This finding was an unintentional result observed while

trying to image retrograde labeled ganglia neurons. When all of the vasculature of the IMG was filled, 99% of the cells observed were positive for both venous and arterial innervation.

Fenestrated capillaries found within ganglia are susceptible to leaking retrograde tracers and causing false positive results. (Baker, 1989;Baluk, 1987). When radioactive cadmium is injected intravenously into rats, the largest areas of accumulation are in the ganglia (Arvidson & Tjalve, 1986). Unlike the blood brain barrier, blood vessels encompassing the IMG which have been filled with fluorescent tracers could be leaking retrograde chemicals out among neurons. These cells could then be phagocytosing the fluorochromes and appear false-positive for blood vessel innervation. The images acquired after injecting two different fluorescent retrograde tracers into the veins and arteries (Figure 2) showed both venous and arterial vessels were in close proximity to the ganglia. Fluorochromes could leak out of the capillary fenestrations around the ganglia causing cells to falsely appear positive for retrograde filling from both venous and arterial blood vessels.

Another finding from our results suggesting retrograde tracers leaking is observed through the chemical Fast Blue. When applied on top of the ganglia only neuronal cells were observed taking up this chemical (Figure 13). When injected into the blood vessels a large number of non-neuronal cells were intensely labeled with Fast Blue, compared to adjacent neurons (Figure 10). Small Intensely Fluorescing (SIF) cells in ganglia have been found to cluster around the fenestrations of ganglionic capillaries (Abe *et al.*, 1983;Matthews,

1989). Non-neuronal SIF cells may be fluorescing as a result of retro-grade tracer uptake from leaking capillaries.

Non-neuronal cells could also fill with fluorochromes as a result of connections between other cells. It has been shown that SIF cells give and receive synaptic connections (Matthews, 1989). Gap junctions have also been observed between neurons and encompassing small satellite cells (Baluk & Fujiwara, 1984;Baluk *et al.*, 1985). Fast blue could move through these small openings where larger retrograde tracing beads might not be able to.

Avoiding the filling of the vasculature encompassing the IMG by injecting into distal blood vessels identified neurons uniquely innervating only veins or arteries. Browning et al, (Browning *et al.*, 1997) using similar retrograde labeling techniques and tissue preparations from guinea pig found similar results. Both Browning's results in the guinea pig (Browning *et al.*, 1997), and our results in the rat, support the existence of separate neuronal pathways for splanchnic venous and arterial blood vessels. In addition we were able to identify a previously un-described blood supply to the inferior mesenteric ganglion.

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