Developmental Cellular Changes in the Superior Cervical Ganglion of Normotensive and Hypertensive Rats

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by
Nega Gerard

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This project would be incomplete but for a heartfelt expression of gratitude to all who made it possible.

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ABSTRACT

Developmental cellular changes in the superior cervical ganglion of hypertensive and normotensive rats

A thesis presented to the Interdepartmental Program in Neuroscience

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Nega Gerard

Primary or essential hypertension is a major risk factor for many cardiovascular diseases. Since it has no identifiable cause, deducing its pathophysiology has been a major research focus in order to understand the underlying mechanisms contributing to high blood pressure. Dysfunction of the cardiac sympathetic nervous system causing sympathetic overdrive in the heart is one such mechanism linked to hypertension. This project was proposed to study the cellular level changes occurring in the superior cervical ganglion (SCG) and its influence on cardiac sympathetic neurotransmission of hypertensive animals. Here, I used the Spontaneously Hypertensive Rat (SHR), an animal model of primary hypertension to study these changes in vivo, at birth (P2), pre-hypertensive (3 weeks) and hypertensive (8 and 12 weeks) states.

I found that SCGs of hypertensive rats have an elevated neuronal density at 3 weeks old which is restored to values comparable to those of normotensive WKY rats by 8 weeks of age. There were no significant differences in the glial cell density and glial neuronal ratio between WKY and SHR SCGs. However, an increase in the glial neuronal ratio was observed with age in both strains which can be correlated with the decrease in their neuronal densities.
On quantifying the glial envelope area estimated by S100β expression, a trend of higher S100β expression in SHRs than in WKY rats at each time point studied was observed with a significant difference in 8 weeks old males. The results on studying glial activation in the SCG showed that 8- and 12- weeks old SHR males exhibit significantly higher GFAP expression compared to age-matched WKY males. This suggests that satellite glial cells are more activated at older ages in hypertensive males than in age-matched controls. These findings showing an increased expression of glial proteins, S100β and GFAP might indicate increased neuron-glial communication within the SCGs of hypertensive animals. Further experiments might validate these findings and future work is needed to elucidate how these observed developmental neuronal and satellite glial cell changes in the SCG and other modulatory factors may contribute to the impaired cardiac sympathetic activity seen in hypertensive animals.
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>BLBP</td>
<td>Brain lipid-binding protein</td>
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<tr>
<td>BW</td>
<td>Body Weight</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HW</td>
<td>Heart Weight</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule Associated Protein 2</td>
</tr>
<tr>
<td>n.s.</td>
<td>Non-significant</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<td>------------------------------------</td>
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<tr>
<td>S100-β</td>
<td>S100 calcium-binding protein B β-subunit</td>
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<tr>
<td>SCG</td>
<td>Superior Cervical Ganglia</td>
</tr>
<tr>
<td>SGC</td>
<td>Satellite glial cells</td>
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<tr>
<td>SHR</td>
<td>Spontaneously Hypertensive Rats</td>
</tr>
<tr>
<td>SIF</td>
<td>Small intensely fluorescent</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>VAChT</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
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</table>
INTRODUCTION

I) Background of hypertension

Hypertension or high blood pressure is a condition where the arterial blood pressure is abnormally high and is one paramount contributing cause of many life-threatening health issues. Recent epidemiological data from the Centers for Disease Control and Prevention (CDC) state that 1 in every 3 adults in the US has hypertension and about 70% of those hypertensive individuals use medications to control high blood pressure (cdc.gov, 2018). The CDC estimates about 800,000 youth aged 12-19 years in the US alone with hypertension as of 2016 (cdc.gov, 2018). Hypertension is known to be more prevalent and severe in males than females until around the age of 60 and thus several studies have focussed on understanding the gender differences in the incidence and severity of hypertension (Everett and Zajacova, 2015; Gillis and Sullivan, 2016).

About 95% of hypertensive individuals have essential or primary hypertension wherein the etiology of high blood pressure is unknown and cannot be attributed to any secondary causes such as renal failure or renovascular disease (Carretero and Oparil, 2000). It is believed to stem from the interaction of various genetic and environmental factors (Bolívar, 2013). Hypertension may result in various complications such as heart attack, heart failure, atherosclerosis, stroke, etc., Moreover, comorbidities in hypertensive patients worsen their health-related quality of life (Zygmontowicz et al., 2012). Since essential hypertension has no direct identifiable cause, it is vital to study how the molecular mechanisms maintaining cardiac homeostasis are perturbed, thereby contributing to the development of hypertension.
II) Overview of the cardiac sympathetic nervous system

The autonomic nervous system, comprised of the sympathetic, parasympathetic and the enteric nervous systems, controls internal organs and thereby regulates bodily functions. The sympathetic nervous system and the parasympathetic nervous system can act independently, synergistically or antagonistically to regulate their target organs (Barman et al., 2016). The cardiac sympathetic nervous system plays crucial roles in increasing heart rate, cardiac output and cardiomyocyte depolarization rates (Esler, 2016). Cardiac sympathetic innervation is supplied by sympathetic nerves that innervate the atria and ventricles of the heart and stimulate increased heart rate and conduction velocity by releasing norepinephrine (Habecker et al., 2016). Despite the significance of their influence in maintaining homeostasis, little is known about the molecular and cellular mechanisms regulating sympathetic activity and the contributing factors altering their activity in pathology. Studies have shown the adverse effects sympathetic system dysfunction has on cardiac function, namely, hypertension, arrhythmias, heart failure, atherosclerosis and sudden cardiac death (Larsen, Lefkimmiatis and Paterson, 2016; Marina et al., 2016). Growing awareness of the importance of sympathetic nervous system activity in blood pressure regulation has resulted in various treatment and management strategies being explored in clinical and translational medicine.

III) Cardiovascular control by peripheral nervous system

Several studies have focused on expounding the contribution of central nervous system mechanisms to increased sympathetic tone. While it is vital to understand the central nervous system control of cardiovascular function, it is equally important to study the peripheral nervous system working in tandem to maintain the cardiovascular homeostasis. Synaptic transmission in the peripheral sympathetic nervous system is facilitated by the sympathetic chain ganglia which consist of sympathetic neuronal cell bodies (Squire, 2008).
Pre-ganglionic fibres from the spinal cord synapse onto sympathetic ganglionic neurons from which the postganglionic fibres extend their axons to the effector organs (Squire, 2008). Pre-ganglionic fibres are cholinergic while post-ganglionic fibres are noradrenergic. The superior cervical ganglion (SCG) is a spindle-shaped, fusiform structure that is part of the paravertebral sympathetic chain ganglia (Barral and Croibier, 2009). It gives rise to the cardiac plexus which innervates the heart (De Gama et al., 2012; Lingford-Hughes and Kalk, 2012). Although majority of the sympathetic innervation to the heart is supplied by the ‘middle cervical-stellate ganglion’ complex, the SCG also, to a lesser extent, supply sympathetic innervation to the heart (Pardini, Lund and Schmid, 1989; Squire, 2008; De Gama et al., 2012). The SCG with its complex neural circuit comprises of neurons (chiefly, noradrenergic neurons), satellite glial cells (SGCs) and small intensely fluorescent (SIF) cells (Hanani, 2010; Takaki et al., 2014).

IV) Characteristics of the cells of the superior cervical ganglion

*Sympathetic neurons* can be identified by their expression of tyrosine hydroxylase (TH) or the cytoskeletal protein, Microtubule Associated Protein-2 (MAP2). These neurons undergo rapid growth and differentiation during embryonic development with their axonal fibers reaching target tissue including the heart as early as E15 (Rubin, 1985). However, postnatally, the high cell numbers decline considerably due to neuron degeneration (Wright, Cunningham and Smolen, 1983). Developmental studies over the years have shown that the cardiac system and the sympathetic nervous system undergo co-development and combined maturation mediated by mutual signaling between the two systems (Habecker et al., 2016). For example, neuronal developmental processes such as axonal outgrowth, neuronal differentiation, target tissue innervation at the perinatal stage is facilitated by cardiac-derived trophic factors, particularly, nerve growth factor (NGF). On reaching the heart tissue, cardiac signals suppress axonal growth of sympathetic axons (Habecker et al., 2016). Such
developmentally timed events of neuronal and axonal growth and degeneration at specific ages are important for the proper overall growth and development of the cardiac-sympathetic nervous system. Although external trophic signals as described above play significant roles in synaptic transmission of sympathetic neurons, signals from surrounding glial cells are also known to modulate sympathetic neuron activity.

**Satellite glial cells (SGCs)** are irregular-shaped glial cells derived from pluripotent neural crest cells that ensheath neurons of the sympathetic ganglia (Costa and Neto, 2015). They express glial-specific markers, GFAP and S100β. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is a marker of glial activation (Feldman-Goriachnik, Wu and Hanani, 2018). S-100 proteins are calcium-binding proteins that regulate Ca\(^{2+}\) homeostasis by modulating the signal transduction pathway (Schäfer and Heizmann, 1996; Marenholz, Heizmann and Fritz, 2004). One of these proteins, S-100β (S-100 calcium-binding protein-β) is a Ca\(^{2+}\) signaling protein that at low concentrations, has trophic effects such as increasing neuronal survival and at high concentrations, has toxic effects such as inducing apoptosis (Higashino et al., 2009; Xia et al., 2018).

Research has shown that SGCs play important roles under normal and pathological conditions and are known to be altered by injury (Feldman-Goriachnik, Wu and Hanani, 2018). A study by Hanani et al., showed that following nerve injury in sensory ganglia, SGC cell-cell coupling increased manifold and resulted in glial activation characterized by increased production of glial fibrillary acidic protein (GFAP) (Hanani et al., 2002). In the sympathetic ganglia, a tremendous reduction in synaptic transmission was observed following nerve injury and it is believed that these SGCs play a role in the process of ‘synaptic stripping’ (selective elimination of damaged neurons and synapses) (Hanani, 2010). A recent paper demonstrated that SGCs in the superior cervical ganglion expressed muscarinic acetylcholine (ACh) receptors and the application of ACh resulted in elevated
intracellular calcium levels and increased GFAP expression (Feldman-Goriachnik, Wu and Hanani, 2018). These findings support the idea that SGCs may influence synaptic transmission in sympathetic ganglia (Hanani, 2010).

V) Neuron-glial cell communication

Neurons and glial cells are known to communicate dynamically from early developmental stages and their interaction is essential for glial cell proliferation, myelination, neuronal migration, synapse elimination and synaptic transmission (Fields, 2002). Neurotransmitters, signalling molecules and ion fluxes act as signals for communication and elicit cellular responses by modulating the neuronal micro-environment (Fields, 2002). Most signals trigger Ca\(^{2+}\) waves as the second messenger system which in turn modulates local neuronal excitability (Marina et al., 2016). In sensory ganglia, it has been shown that peripheral injury results in neuronal release of ATP which activates the SGC purinergic receptors by increasing Ca\(^{2+}\) levels in neighbouring SGCs. This, in turn, leads to release of inflammatory cytokines that can modulate neuronal excitability (Costa and Neto, 2015). This and several other studies provide evidence that calcium waves are a major means of signal propagation between neurons and glial cells (Haydon, 2000; Fields, 2002; Costa and Neto, 2015).

VI) Experimental animal model: The spontaneously hypertensive rat (SHR)

A strain of the spontaneously hypertensive rat (SHR) was developed by Okamoto and Aoki by selective inbreeding of Wistar Kyoto (WKY) rats with high blood pressure (Okamoto and Aoki, 1963). The spontaneously hypertensive rat is known to develop left ventricular hypertrophy early in life before becoming spontaneously hypertensive within 15 weeks of age (Okamoto and Aoki, 1963; Tanase, 2018). The SHR is a widely employed animal model for hypertension since it closely resembles human primary hypertension and
was adopted in this study (Pinto, 1998; Tanase, 2018). The normotensive Wistar Kyoto rats were used as controls in this study.

VII) Dysfunction of the cardiac-sympathetic system in spontaneously hypertensive rats

A study by Li et al. (2012) showed that cultured neurons from the superior cervical ganglion of SHRs demonstrated larger calcium transients compared to that of WKY rats (Li et al., 2012; Shanks et al., 2013). Degeneration of sympathetic fibers, elevated Ca\(^{2+}\) transients, impaired noradrenaline (NA) uptake and enhanced NA release all contribute to enhanced cardiac sympathetic neurotransmission in SHRs (Li et al., 2012; Shanks et al., 2013). Since calcium induces norepinephrine (NE) release and NE increases heart rate and blood pressure, the abnormal Ca\(^{2+}\) homeostasis seen in SHRs might contribute to the observed increased cardiac sympathetic drive (Li et al., 2012).

Ca\(^{2+}\) homeostasis dysregulation was observed at birth, prehypertensive and at hypertensive ages in SCGs of SHR animals (Li et al., 2012). Our lab has recently shown that SHR animals have increased cardiac sympathetic innervation even at birth and in the early postnatal period (i.e., P2, P7, P14, P28) (Huang, 2018, unpublished). Taken together, these studies suggest that the cardiac sympathetic neurotransmission in SHR animals is impaired well before the onset of hypertension. To understand the causal molecular basis of this disruption seen in hypertensive animals, we thus sought to look at the changes occurring at the cellular level in the SCGs of hypertensive and normotensive animals across different ages. Understanding such cellular and morphological changes may shed light on the pathophysiological mechanisms resulting in the development of hypertension.
Objective

➢ To study and compare the cellular composition changes in the superior cervical ganglion (SCG) of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats at different developmental time points.

➢ To elucidate the modulatory role of satellite glial cells in cardiac sympathetic neurotransmission of the superior cervical ganglion by studying expression of glial proteins, S100β and GFAP.
MATERIALS AND METHODS

Animals

The Spontaneously Hypertensive Rats (SHR) and the Wistar Kyoto (WKY) rats were first received from Charles River Laboratories. The animals were housed and bred in the Foster lab animal facility, Brandeis University.

Ethical approval

Animals were euthanized by CO₂ asphyxiation and SCG tissues were obtained from WKY rats and SHRs in accordance with the Brandeis University Institutional Animal Care and Use Committee.

Tissue extraction

The SCG tissues were isolated from WKY rats and SHRs - aged P2, 3 weeks (P21), 8 weeks (P56) and 12 weeks old (P84). Tissues from male and female rats of both strains at 8 weeks and 12 weeks old were processed separately. The isolated SCGs were fixed for at least overnight in 4% paraformaldehyde (PFA) at 4°C. Following fixation, the tissues were washed thrice with Phosphate Buffer Saline (PBS) for 15 minutes and then cryo-protected by incubating them in 30% sucrose solution at 4°C for at least overnight or until the tissues sank.

Tissue processing

The tissues were then placed in cryo-molds and embedded with O.C.T (optimal cutting temperature) compound (Tissue-Tek O.C.T Compound, Sakura Finetek, VWR, CA, USA) and orientated before freezing them using dry ice. SCGs were immersed in TrueBlack
solution (TrueBlack® Autofluorescence Quencher) for a few seconds before O.C.T embedding for easy identification and the embedded tissues were stored at -80°C until use. The tissues were cut into 10μm, longitudinal sections in a cryostat (Leica CM3050, Buffalo Grove, IL, USA) and thaw mounted onto Fisherbrand™ ColorFrost™ Plus Microscope Slides. The slides with the cryosections were stored at 4°C until use.

**Immunohistochemistry**

Appropriately matched slides were chosen and rehydrated in PBS for 30 minutes and the boundary of each section was marked using the ImmEdge Hydrophobic Barrier Pen (Vector Labs, Inc., Burlingame, CA). Slides were then placed on ice and treated thrice with 10mg/mL sodium borohydride solution for 10 minutes each time to reduce autofluorescence. The slides were then washed thrice with PBS for 10 minutes each time. Subsequently, slides were immersed in the blocking buffer (3% Bovine serum albumin (BSA)/0.3% Triton X-100 in PBS) and incubated for 1 hour in a shaker. SCG sections were incubated overnight at room temperature in a sealed humidified chamber with primary antibodies at the following concentrations: chicken anti-Microtubule Associated Protein 2 (MAP2) polyclonal antibody (Sigma-Aldrich, EMD Millipore, Darmstadt, Germany, AB5543, 1:1500); rabbit anti-S100 calcium-binding protein B β-subunit (S100-β) polyclonal antibody (Agilent Dako, Santa Clara, CA, Z0311, 1:400) or rabbit anti-GFAP antibody (BioLegend, San Diego, CA, 840001, 1:1000) at room temperature. The preliminary staining shown in Figure 10 was performed using the following primary antibodies: chicken anti-tyrosine hydroxylase (TH) polyclonal antibody (Abcam, USA, AB76442, 1:1000), rabbit anti-vesicular acetylcholine transporter (VACHT) polyclonal antibody (Synaptic Systems, Goettingen, Germany, VACHT 139 103, 1:200). The excess unbound antibodies were washed away by rinsing the slides 3 times with 1X PBS for 10 min each time. Following the washing step, the sections were incubated with donkey anti-chicken rhodamine and donkey anti-rabbit Alexa 488 secondary
antibody for 1.5 hours at room temperature under dark conditions. They were then rinsed once with 1X PBS for 10 min while maintaining dark conditions to remove any unbound antibodies and prevent non-specific binding. Subsequently, the SCG slides were incubated with 1 mg/ml 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen Life Technologies) (1:20) for 15 mins and were washed with PBS twice for 10 min each time in the dark. They were then immersed briefly in milli-Q H₂O. The excess H₂O was then wiped off and PBS was added to the slides before adding mounting medium. Then, glass coverslips were mounted on the slides using about 40μl of 1:1 glycerol: 1X PBS mounting solution and were sealed with nail polish.

**Imaging and image analysis**

The stained SCG sections were then imaged using the Zen software (Zeiss) on Zeiss LSM 880 laser scanning confocal microscope and analyzed for neuron density, glial envelope area fraction, glial cell density and GFAP mean intensity fold change in SHR and WKY rats. Images of SCG sections of P2 and 3 weeks old animals were captured using a 40x objective and those of 8 weeks old and 12 weeks old animals were captured using a 20x objective.

(i) **Neuron density, glial cell density and glial envelope area fraction**

3 regions of interest (ROI) were chosen per section and 3 sections per animal were imaged using the 561nm, 488nm and 405nm lasers to excite the three fluorochromes: rhodamine, Alexa 488, and DAPI, respectively. Neurons in SCG sections were identified by MAP2 staining, glial cells by S100-β staining, and nuclei by DAPI staining. Cells with MAP2 staining were counted as neurons and the number of neurons was calculated using the Cell Counter plug-in of the Fiji (SciJava Consortium) software. The neuronal density was calculated by dividing the total number of neurons in the imaged ROI by the area of that ROI.
Cells stained for both S100β and DAPI were identified and counted as glial cells. The number of glial cells was calculated using a MATLAB script (Author: Alexander Mitchell, Birren Lab) (see Appendices: Appendix I) and the results were compared. The glial cell density was then calculated by dividing the estimated number of glial cells in the imaged ROI by the area of that ROI. The glial envelope area fraction or the S100β expression area fraction was determined by dividing the area of S100β expression by the total area of the imaged ROI. The area of S100β expression was measured using Fiji. All data were pooled from 3 animals for each age and strain and/or gender.

(ii) Average GFAP intensity fold change

SCG sections were stained with MAP2, GFAP and DAPI to visualize activated glia. The mean GFAP intensity was determined using Fiji and the fold change of the mean GFAP intensity normalized to the control (WKY) was calculated for each age and age/gender. Representative images (displayed in the Results section) were enhanced by adjusting Brightness/Contrast (B/C) using Color Balance tool in ImageJ. Identical image processing was performed on all displayed images of both strains and all ages for better visualization and representation purposes only.

Statistical analysis

All data presented are the mean data obtained from 3 animals for each age and strain and/or gender. Student’s t-test was performed to compare results and significance level and the test values (p) are provided. Data were consolidated and analyzed using Microsoft Excel 2010 software and graphs were rendered using GraphPad Prism 8.
RESULTS

I) Developmental cellular composition changes in SCGs of WKY rats and SHRs

In order to study the developmental cellular changes occurring in the SCGs of WKY and SHR animals, the SCGs of P2, 3 weeks old and 8 weeks old animals were analyzed for neuronal and satellite glial cell density. Figures 1 and 2 show the SCGs of the two strains at different ages stained for the neuronal marker: MAP2, glial cell marker: S-100β and cell nuclei (DAPI).

**SHR animals have higher neuronal density at the pre-hypertensive state**

The sympathetic neurons were stained for the neuron-specific marker, MAP2 (red) and cell nuclei using DAPI (blue). It can be seen that each neuron is enwrapped by many satellite glial cells as observed here (Figs. 1, 2) and in previous studies (Hanani, 2010). Plot of the quantified neuronal densities of WKY rats and SHRs is displayed in Figure 3. There were no significant differences in the neuron density between the two strains at P2 (4046.85/mm² ± 149.17 vs 3820.89/ mm² ± 31.45, n=3, p=n.s.). However, the neuronal density of 3 weeks old SHR SCGs was found to be significantly higher than the age-matched WKY SCGs (1119.57/mm² ± 42.05 vs 1496.86/ mm² ± 23.87; n=3, p= 0.0036). At 8 weeks old, it was found that there was no significant difference in the neuronal densities of both male (624.18/ mm² ± 36.35 vs 684.13/mm²± 61.49, n=3, p=n.s.) and female (769.19/mm² ± 44.73 vs 851.78/ mm² ± 19.75, n=3, p=n.s.) SCGs of WKY and SHR animals. These results show that the higher neuronal density observed in SHR animals at 3 weeks old is restored to comparable values of WKY animals by 8 weeks of age.
Figure 1. SCGs of P2 and 3 weeks old WKY rats and SHRs. Representative confocal merged images at 40x magnification of WKY (left) and SHR (right) SCGs with MAP2 staining (red), S100-β staining (green) and DAPI staining (blue) from P2 pups and 3 weeks old animals (scale bar=60μm).
Figure 2. SCGs of 8 weeks old WKY rats and SHRs. Representative confocal merged images at 20x magnification of WKY (left) and SHR (right) SCGs with MAP2 staining (red), S100-β staining (green) and DAPI staining (blue) from 8 weeks old male and female animals (scale bar=60μm).
Figure 3. Sympathetic neuronal density changes in SCGs of WKY rats and SHRs at different developmental time points. Bar plot of the neuronal density of WKY and SHR SCGs at P2, 3 weeks old and 8 weeks old (male and female). (n=3; Error bars, SEM). Unpaired Student’s t-test was conducted for each age and age/gender group (***p<0.01).

**SCG glial neuronal ratio is likely to vary with neuronal density and not glial cell density**

Satellite glial cells were stained for S100-β (green) and cell nuclei using DAPI (blue). Figures 1 and 2 show that the SGCs form a continuous sheath around the neuronal cell bodies. On analyzing the average glial cell density data of WKY and SHR SCGs, no significant differences were found at P2 (4616.25/mm² ± 540.33 vs 3512.09/mm² ± 606.69; n=3, p= ns), 3 weeks old (5017.66/mm² ± 559.26 vs 5267.54/mm² ± 525.37; n=3, p= ns), 8 weeks (males) (3642.48/mm² ± 187.43 vs 3752.03/mm² ± 114.97; n=3, p= ns) and 8 weeks (females) (2994.64/mm² ± 495.51 vs 3997.05/mm² ± 207.94; n=3, p= ns) (Fig. 4A). In contrast to the neuronal density that declined considerably postnatally, the glial cell density remained relatively comparable between WKY and SHR animals throughout the ages studied.
Figure 4. Satellite glial cell density and glial neuronal ratio changes in SCGs of WKY rats and SHRs  
A) Bar plot of the glial cell density of WKY and SHR SCGs at P2, 3 weeks old and 8 weeks old (male and female) (n=3; Error bars, SEM).  
B) Bar plot of the glial neuronal ratio of WKY and SHR SCGs at P2, 3 weeks old and 8 weeks old (male and female) (n=3; Error bars, SEM). Unpaired Student’s t-test was conducted for each age and age/gender group.
Quantification of the glial neuronal ratio in WKY and SHR SCGs is shown in Figure 4B. There were no significant differences in the glial neuronal ratio between the two strains at P2 (1.15 ± 0.14 vs 0.92 ± 0.17, n=3, p=n.s.), 3 weeks old (4.49 ± 0.54 vs 3.53 ± 0.41, n=3, p=n.s.), 8 weeks old (males) (5.88 ± 0.48 vs 5.48 ± 0.58, n=3, p=n.s.) and 8 weeks old (female) (3.89 ± 0.75 vs 4.69 ± 0.19; n=3, p=ns) (Fig. 4A). These results show that the increase in the glial neuronal ratio with postnatal development observed in both strains is likely due to decrease in their neuronal densities rather than their glial densities which remains relatively similar across ages.

II) Modulatory role of satellite glial cells in sympathetic neurotransmission of the SCG

Recent studies show evidence that satellite glial cells play important roles in pain, inflammation and modulating neuronal activity (Hanani, 2010). We wanted to study whether satellite glial cells modulate sympathetic neuron activity in the superior cervical ganglion. In order to do so, we analyzed the expression of glial proteins, S100β and GFAP.

**SCGs of SHR males exhibit significantly higher S100β expression at the hypertensive state**

The glial envelope in the SCGs was defined by S100β expression of satellite glial cells in this study. The glial envelope area estimated by the S100β expression area fraction in WKY and SHR SCGs at different ages is shown in Figure 5. It was found that the S100β expression area fraction is higher in the SHRs at P2 (0.533 ± 0.043 vs 0.630 ± 0.005, n=3, p=n.s.), 3 weeks old (0.635 ± 0.07 vs 0.763 ± 0.044, n=3, ns) and significantly higher at 8 weeks old (0.622 ± 0.014 vs 0.757 ± 0.043, n=6, p=0.0235) compared to the controls (Fig. 6A). A comparison of S100β expression area fraction in 8 weeks old males (0.640 ± 0.021 vs 0.837 ± 0.039, n=3, p=0.0199) and females (0.603 ± 0.014 vs 0.677 ± 0.154, n=3, p=0.0235) of both strains was done to detect gender-wise changes in the S100β expression (Fig. 6B). It can be seen that 8 weeks SHR males show a significantly higher S100β expression than the WKY rats compared to the females.
Figure 5. S100β staining of SCGs from P2, 3 weeks- and 8 weeks old WKY rats and SHRs. Representative confocal merged images of WKY (left) and SHR (right) SCGs with S100-β staining (green) and DAPI staining (blue) from P2 (at 40x magnification), 3 (at 40x magnification) weeks and 8 weeks old (male and female) animals (at 20x magnification) (scale bar=60μm).
Figure 6. Glial envelope area marked by immunohistochemical expression of S100β in SCG of WKY rats and SHRs. A) Bar plot of the glial envelope area fraction of WKY and SHR SCGs at P2, 3 weeks old and 8 weeks old (Error bars, SEM). B) Bar plot of the glial envelope area fraction of WKY and SHR SCGs at P2, 3 weeks old and 8 weeks old (male and female) (n=3; Error bars, SEM). Unpaired Student’s t-test was conducted for each age and age/gender group (*p<0.05).
In order to further understand the glial signals modulating cardiac-sympathetic neurotransmission, we wanted to compare satellite glial cell reactivity in SCGs of normo- and hypertensive rats by studying their GFAP expression.

**SHR glial cells are more activated at hypertensive ages compared to age-matched controls**

Immunohistochemical staining of SCG sections from WKY and SHR animals for GFAP was performed to study glial activation. GFAP expression in glial filaments surrounding the neurons in both WKY and SHR SCGs can be observed in Figure 7. Figure 8 shows the plot of fold change of average GFAP intensity of SHR SCGs normalized to WKY SCGs at different ages. At P2, there was no observable GFAP expression; at 3 weeks old, there was a 2-fold mean intensity increase in the SHRs (2.003 ± 0.307, n=3, p=ns (0.0821)). There was not a significant fold change at 8 weeks (1.377 ± 0.187, n=6, p=ns) and at 12 weeks (males), a significant fold change was observed (1.571 ± 0.042, n=3, p=0.0054).

**Figure 7. Immunohistochemical staining for GFAP in the SCG.** Representative confocal images at 63x magnification A) with MAP2 staining (red), GFAP staining (green) and DAPI staining (blue) B) with GFAP staining in SCGs of 12-week old SHR animals. GFAP expression is clearly visible in glial filaments surrounding the neurons (white arrows) (scale bars=30μm).
However, analysing data from 8 weeks old males and females separately revealed a gender-wise difference in GFAP intensity levels. A significant fold change of GFAP intensity was observed in 8 weeks old SHR males (1.598 ± 0.134, n=3, p=0.0469) and not in females (1.157 ± 0.330, n=3, p=ns); and in 12 weeks old SHR males (1.571 ± 0.042, n=3, p=0.0054).

Figure 8. Comparison of GFAP expression in SCGs of WKY rats and SHRs at different ages. A) Bar plot of the average GFAP intensity fold change in WKY and SHR SCGs at P2, 3 weeks, 8 weeks and 12 weeks old (Error bars, SEM). B) Bar plot of the GFAP intensity fold change of WKY and SHR SCGs at P2, 3 weeks old, 8 weeks old (male and female) and 12 weeks old (male) (n=3; Error bars, SEM). Unpaired Student’s one sample t-test was conducted for each age and age/gender group (*p<0.05, **p<0.01).
Figure 9. Immunohistochemical staining of SCGs from 3 weeks-, 8 weeks- and 12 weeks old WKY rats and SHRs SCG for GFAP. Representative confocal images of WKY (left) and SHR (right) SCGs with GFAP staining (green) from 3 weeks old (at 40x magnification), 8 weeks old male and 12 weeks old male (at 20x magnification) animals (scale bar=60μm). Representative images were enhanced by adjusting B/C and identical image processing was performed on all images for better visualization and representation purposes only.
DISCUSSION

There is substantial evidence associating altered sympathetic activity with development and progression of primary hypertension (Marina et al., 2016). Abnormalities occurring at different levels of the sympathetic system both at the afferent and efferent limbs have been studied over the years. Understanding the CNS circuitry has been of prime importance with research showing dysfunction of cardiovascular afferents and altered activity of CNS neuronal circuits contributing to increased efferent sympathetic activity (Marina et al., 2016). However, studying the sympathetic efferent pathway and its alteration will provide insights on the final stages of regulation and dysregulation of received CNS information prior to transmission to effector organs. In this ongoing project, we seek to study the developmental cellular changes occurring in the superior cervical ganglion which is part of the efferent sympathetic system that innervates the heart. In doing so, we want to understand sympathetic dysregulation in hypertensive animals.

Here, first, we wanted to address if the density of neurons and glial cells in SHR animals is altered. Next, in order to study how local glial signals modulate the SCG neuronal microenvironment, we sought to look at the immunohistochemical expression of S-100β in SHR and WKY animals. Finally, building on previous research (see Introduction), we wanted to investigate whether satellite glial cells in the peripheral sympathetic ganglia are more activated in the SHR animals than in the WKY animals.

The results in Fig. 3 show that postnatally, the neuronal density declines dramatically and then rather slightly from 3- to 8- weeks old in both WKY and SHR animals. It can be seen that following birth, the neuronal density is relatively similar in WKY and SHR animals
but at the pre-hypertensive age (3 weeks old), there is a significant difference between their neuronal densities. The higher neuronal density seen in 3 weeks old SHRs compared to age-matched WKY rats could be due to a smaller neuronal size or due to less neuronal degeneration in the SHR animals. Interestingly, the neuronal density of both strains equalizes at 8 weeks old in both male and female rats. This suggests that certain events occurring between the ages of 3-8 weeks old in hypertensive animals restore neuronal densities (to values comparable to those of normotensive WKY rats) by adulthood. There was no significant difference in the average glial cell density and the glial neuronal ratio between WKY and SHR SCGs at P2, 3 weeks old and at 8 weeks (Fig. 4). However, the increasing trend of the glial neuronal ratio with postnatal development can be correlated with the decrease in the neuronal density since the glial density was found to be relatively comparable across ages. Quantification of the neuron soma size will further elucidate these findings.

There were no significant differences in the glial envelope area (area fraction of S100β expression) between WKY and SHR SCGs at P2 and 3 weeks old, but a significant difference was found at 8 weeks old (males) and overall, a trend of increased S100β expression in the SHRs compared to the WKY rats was observed at all ages (Fig. 6A). Since the glial density remains relatively similar in WKY and SHR SCGs, the observed trend of higher expression of the calcium binding protein, S100β in SHRs at each age studied is likely due to increased expression of the protein itself. Since it is known that glial cells modulate neuronal activity by Ca2+ waves, these results suggest that glia may be more dynamic in the SHRs than in the WKY and may indicate increased neuron-glia communication via Ca2+ signalling. However, quantifying S100β mRNA and protein levels would be necessary to verify that the above deduction is a physiological reality. Studying the effect of exogenous application of S100β in the SCG would address if S100β is involved in glial-sympathetic neuron Ca^{2+} signaling mechanisms within the SCG.
It is well known that males are more likely to develop hypertension than females and researchers have long tried to elucidate the mechanisms underlying this gender differences in hypertension. This difference is seen even in SHR animals where the males develop high blood pressure earlier and more severely than females (Sandberg and Ji, 2012). To account for the influence of these gender differences in observed results, males and females were studied separately for hypertensive ages of the animal model. The significant change in S100β expression observed in 8 weeks old SHR males to WKY males compared to the females suggests that alteration of glial signals may be exacerbated or occurs earlier in the males than in the females (Fig. 6B). Studying these neuronal and glial changes at older ages may provide insights on these observations and speculations.

Recent studies demonstrating increased ATP levels and thereby increased Ca\textsuperscript{2+} levels due to astroglial activation show evidence of abnormal glial activity contributing to increased sympathetic tone (Marina et al., 2016). In order to explore in depth how such glial signals might regulate neuronal activity in the peripheral sympathetic system, we studied satellite glial cell activation in SHR animals. The results in Figure 8A show significant glial activation in SHR animals compared to WKY in 8 weeks old males and 12 weeks old males. These findings suggests that satellite glial cells in the sympathetic ganglion may be responding to received signals from neighboring neurons and may in turn influence the neuronal microenvironment. However, to reduce the influence of tissue section variability in the GFAP intensity data, more replicates need to be conducted to validate the above findings. In the future, estimating number of neurons surrounded by GFAP\textsuperscript{+} glia would be a more accurate method of analysis.
Recent \textit{in vitro} work from our lab suggests that dysfunction of SHR myocytes may contribute to sympathetic hyperinnervation seen in the SHRs, possibly by increased release of trophic factors. In light of the above results, it can be hypothesized that increased trophic signals from the heart in SHR animals during the postnatal period might result in the elevated neuronal density seen at the pre-hypertensive state (3 weeks old). This might drive increased glial signalling and activation evident from the above findings. However, continued increase in glial signalling might possibly contribute to increased sympathetic neuronal activity in hypertensive animals.

In summary, the study suggests that perturbation of neuronal density in the superior cervical ganglion at the pre-hypertensive state might trigger modulatory glial signals and sustained increase of these signals with development might in turn affect neuronal activity in hypertensive animals. These findings suggest that satellite glial cells might modulate neuronal activity in hypertensive animals. Future research can validate the observed findings and elucidate whether and how increased neuron-glia signaling within the SCG might contribute to increased sympathetic drive and thus, sympathetic hyperactivity in hypertensive animals.
CONCLUSIONS AND FUTURE DIRECTIONS

The above study has focused on understanding the neuronal and satellite glial cell changes occurring in the superior cervical ganglion of hypertensive animals and how glial signals in the SCG might modulate sympathetic neuronal activity. The findings signal a possible correlation between the cellular level changes in the SCG and increased cardiac sympathetic hyperactivity in hypertensive animals. Future work in the following areas might advance the current understanding of such cellular level modifications observed in hypertensive animals:

- It is known that application of acetylcholine results in depolarization of the superior cervical ganglion (Koketsu and Nishi, 1968). This suggests that larger depolarization triggered by increased ACh release may be driving the enhanced NE release seen in SHR animals (Li et al., 2012). Thus, it would be interesting to test this hypothesis in the future in SHR and WKY animals. Figure 10 from a preliminary experiment shows the immunohistochemical staining of VAChT puncta in the superior cervical ganglion of 8 weeks old WKY and SHR animals. The preliminary staining revealed a higher VAChT puncta/neuron ratio in SHR animals compared to the age-matched WKY rats. Replicate experiments will need to be performed to quantify the VAChT staining and to see if consistent results are obtained. Future IHC experiments to study other cholinergic markers at different developmental time points may provide insights on developmental changes of cholinergic signaling in WKY and SHR animals.
Figure 10. VAChT staining of SCGs from 8-week-old male WKY rats and SHRs.
Representative confocal images at 20x magnification of (top) VAChT staining (green); and
(bottom) TH staining (red), VAChT staining (green) and DAPI staining (blue) of SCG of
WKY (left) and SHR animals (right) at 8 weeks old (scale bar=60μm).
There are numerous studies demonstrating that glial cells communicate via gap junctions facilitating cell coupling (Hanani, 2005). Gap junction-mediated satellite glial cell coupling in sensory ganglia has been shown to increase multiple-fold upon nerve injury (Hanani et al., 2002). An increase in the immunoreactivity of the gap junction protein, connexin-43 has also been observed after nerve injury (Hanani et al., 2002). Knowing that connexin proteins are primary components of gap junctions, it would be interesting to visualize the immunohistochemical localization of these proteins in the superior cervical ganglion and study their expression levels in WKY and SHR animals. This would enable us to elucidate the signal propagation facilitated by Ca\textsuperscript{2+} waves seen in neurons and satellite glial cells of the sensory ganglia in sympathetic ganglia. Given that in contrast to sensory ganglionic neurons, sympathetic ganglionic neurons receive synaptic inputs and that sympathetic satellite glial cells closely ensheath neurons, emphasizes the need for more work in this topic (Hanani, 2010).

Research in these specific areas would shed further light on the cellular and molecular mechanisms contributing to the pathophysiology of hypertension.
Glial cell number was estimated using the following MATLAB script:

**Author: Alexander Mitchell, Birren Lab**

```matlab
%tic;
%Run for SHR to begin with at 8wks
thresh_SHR = 15;
thresh_WKY = 15;

%These are the names of the folders that contain the images
SHR_Name = 'SHR';
WKY_Name = 'WKY';

[SHR_FileNames,SHR_MeanIntensity,SHR_TotalGreenIntensity,SHR_NumberofGreenPixels,SHR_allImages,SHR_greenImage,SHR_RedandGreenSimilarity] = RunGreenGreens(SHR_Name,thresh_SHR);

%change to WKY 8wks
[WKY_fileNames,WKY_MeanIntensity,WKY_TotalGreenIntensity,WKY_NumberofGreenPixels,WKY_allImages,WKY_greenImage,WKY_RedandGreenSimilarity] = RunGreenGreens(WKY_Name,thresh_WKY);

[h_mi,p_mi,ci_mi,stats_mi] = ttest2(SHR_MeanIntensity,WKY_MeanIntensity);
[h_gi,p_gi,ci_gi,stats_gi] = ttest2(SHR_TotalGreenIntensity,WKY_TotalGreenIntensity);
[h_gp,p_gp,ci_gp,stats_gp] = ttest2(SHR_NumberofGreenPixels,WKY_NumberofGreenPixels);

%images are consistently 2048x2048x3
maxSize = 2048*2048;
WKY_meanPixels = mean(WKY_NumberofGreenPixels)/maxSize
SHR_meanPixels = mean(SHR_NumberofGreenPixels)/maxSize

WKYIntensity = mean(WKY_MeanIntensity)./255;
SHRIntensity = mean(SHR_MeanIntensity)./255;

l1 = length(SHR_MeanIntensity);
l2 = length(WKY_MeanIntensity);
N_images = max(l1,l2);

[SHR_nukeSize,SHR_nukeArea,SHR_numCells,SHR_blues] = FindNuclei(SHR_greenImage,SHR_allImages);
[WKY_nukeSize,WKY_nukeArea,WKY_numCells,WKY_blues] = FindNuclei(WKY_greenImage,WKY_allImages);
```
AllData = cell(N_images,10);
AllData(1:11,1) = SHR_FileNames;
AllData(1:11,2:5) = num2cell([SHR_MeanIntensity,SHR_TotalGreenIntensity,SHR_NumberofGreenPixels
 ,SHR_RedandGreenSimilarity]);
AllData(1:11,6:8) = num2cell([SHR_nukeSize,SHR_nukeArea,SHR_numCells]);
AllData(1:12,9) = WKY_fileNames;
AllData(1:12,10:13) = num2cell([WKY_MeanIntensity,WKY_TotalGreenIntensity,WKY_NumberOfGreenPixels
 ,WKY_RedandGreenSimilarity]);
AllData(1:12,14:16) = num2cell([WKY_nukeSize,WKY_nukeArea,WKY_numCells]);

headers = {
'SHR_fileNames',
'SHR_Intensities',
'SHR_GreenIntensity',
'SHR_GreenPixels',
'SHR_scores',
'SHR_nukeSize',
'SHR_nukeArea',
'SHR_numCells',
'WKY_fileNames',
'WKY_Intensities',
'WKY_GreenIntensity',
'WKY_GreenPixels',
'WKY_scores',
'WKY_nukeSize',
'WKY_nukeArea',
'WKY_numCells'};

XLT = cell2table(AllData,'VariableNames',headers);
xlName2 = strcat(SHR_Name,'+',WKY_Name,'_greens_',date,'.xlsx');
writetable(XLT,xlName2);

%---------------------------------------------
%
%RunGreenGreens
%function
%[fileNames,meanIntensity,GreenIntensity,GreenPixels,allImages,greenImage,simScores] = RunGreenGreens(dirName,threshold)
%dirName = 'WKY 8wks';
%threshold = 0;
%cd(dirName)

dirk = dir('*.tif');
N_images = numel(dirk);

fileNames = cell(N_images,1);
allImages = cell(N_images,1);
GreenPixels = zeros(N_images,1);
GreenIntensity = zeros(N_images,1);
greenImage = cell(N_images,1);
simScores = zeros(N_images,1);

for k = 1:N_images
  imago = imread(dirk(k).name);
  if size(imago,3) ~= 3
    imago = imago(:,:,1:3);
  end
  allImages(k) = imago;
  dirk(k).name
  fileNames(k) = dirk(k).name;
  G = imago(:,:,2);
  G0 = G; %original G
  if threshold ~= 0 %very fast operation, threshold really should be 0
    G(G <= threshold) = 0;
  end
%disp(size(imago))
Gt = G >= threshold;
GreenPixels(k) = nnz(G);
GreenIntensity(k) = sum(sum(G));
greenImage{k} = Gt;
figure()
montage({imago,Gt})

cd ..

%Create new folder if it doesn't already exist
%pwd is the current file. In more complicated programs, using
%this can be a very bad idea. For our purposes, this limited usage will
%be acceptable.
currentFolderName = pwd;
newSubFolderName = strcat(dirName,' Greens');
ewSubFolder = [currentFolderName,'/',newSubFolderName];

if ~exist(newSubFolder, 'dir')
    mkdir(newSubFolder);
end
cd(newSubFolderName)
imwrite(Gt, strcat(fileNames{k}(1:end-4),'.png'))
cd ..
cd(dirName)

simScores(k) = ssim(imago(:,:,1),imago(:,:,2));
end
cd ..

meanIntensity = GreenIntensity./GreenPixels;
save(strcat(dirName,'-Green-',date));
end

-----------------------------------------------------------------------------------------------------

function [nukeSize,nukeArea,numGlia,blues] = FindNuclei(greens,images)
%FindNuclei
%Runs NukeFinder to give us the number of glia cells

%greens is a cell array, with each cell containing a logical image
%detailing where there was sufficient green signal.

%images are the original images

blueThresh = 150;  %minimum value to count as a glial nucleus,
%image based thresholding

blues = cell(size(images));
N_images = length(images);
nukeSize = zeros(size(images));
nukeArea = zeros(size(images));
numGlia = zeros(size(images));

for l = 1:N_images
    imago = images{l};
    B = imago(:,:,3);
    B2 = B > blueThresh;
    B3 = B2 & greens{l};
    blues{l} = B3;
    [nukeSize(l),nukeArea(l),numGlia(l)] = NukeFinder(B3,0);
    % figure()
    % imshow(B3)
end
end
REFERENCES


