Evaluation of contribution of cardiac sympathetic innervation to the development of essential hypertension

Master's Thesis

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by
Wenqi Fu

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ABSTRACT

Evaluation of contribution of cardiac sympathetic innervation to
the development of essential hypertension

A thesis presented to the Graduate Program in Molecular and Cell Biology

Graduate School of Arts and Sciences
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Essential hypertension or primary hypertension is defined as manifestation of high blood pressure without secondary reasons, which might be partially related to cardiac sympathetic hyperinnervation (Coote & Chauhan, 2016). Here, I present evidence that early chemical sympathectomy can reverse the development of essential hypertension in adult spontaneously hypertensive rats (SHR). Morphologically, in immunohistochemically stained hearts I observed and quantified changes in cardiac sympathetic fiber density of the left ventricle, and changes in cellular density in developing superior cervical ganglion (SCG). Physiologically, I measured the blood pressure using a non-invasive tail-cuff method and assayed the heart/ body weight ratio. I found that neonatal sympathectomy induced by 6-hydroxydopamine (6-OHDA) diminished cardiac sympathetic innervation and reduced blood pressure of 12-week-old SHR. This indicates existing bonds between cardiac sympathetic hyperinnervation and promoted development of hypertension. Taken together, this study provides evidence that early neonatal intervention directed towards modulation of sympathetic cardiac innervation can influence a course of essential hypertension development.

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List of Abbreviations

1. ATP, Adenosine triphosphate
2. ANOVA, Analysis of variance
3. BP, Blood Pressure
4. BW, Body Weight
5. DAPI, 4′,6-diamidino-2-phenylindole
6. GFAP, Glial fibrillary acidic protein
7. HW, Heart Weight
8. IACUC, Institutional Animal Care and Use Committee
9. IHC, Immunohistochemistry
10. MAP2, Microtubule-associated protein 2
11. nNOS, neuronal Nitric oxide synthase
12. OCT, Optimal cutting temperature
13. 6-OHDA, 6-hydroxydopamine
14. P, postnatal day
15. PBS, Phosphate-buffered saline
16. PFA, Paraformaldehyde
17. SNS, Sympathetic nervous system
18. SCG, Superior cervical ganglion/ganglia
19. SHR, Spontaneously hypertensive rats
20. SD, Sprague Dawley

21. S100 β, S100 calcium-binding protein β

22. WKY, Wistar-Kyoto

Introduction

1) Epidemiology and causes of essential hypertension

Essential hypertension, or primary hypertension, refers to lasting high blood pressure without an identifiable secondary cause. It can be caused by both genetic and environmental factors.

World-wide, about 90-95% of adult hypertensive patients have essential hypertension (Benjamin et al., 2017). Despite relentless efforts in exploring mechanisms in etiologies of essential hypertension, causes are still not completely understood. Nevertheless, there is mounting evidence that the sympathetic nervous system (SNS) plays a critical role in the development of essential hypertension (Wyatt & Textor, 2018).

2) The SNS and its involvement in regulation of essential hypertension

The SNS is one of the two branches of autonomic nervous system, the parasympathetic nervous system being a second, that coordinately maintain blood pressure homeosis (Lee R M et al., 1987). Anatomically, preganglionic neurons of the SNS originate from the middle and lower part of spinal cord, and project their axons to nearby sympathetic ganglia, such as superior cervical ganglia (SCG). The SCG are made up of two cell types: sympathetic neurons and satellite glia. Postganglionic neurons of the SNS then innervate internal organs (such as heart) and vasculature, thus providing sympathetic drive to the tissue. Functionally, the SNS regulates maturation of cardiac tissue and cardiac properties in adulthood (Kreipke & Birren, 2015). Since cardiac output is one of the determinants of blood pressure, by controlling cardiac characteristics and function, the SNS can regulate blood pressure. Emerging evidence suggests that elevated sympathetic drive contributes to the pathological state of hypertension (Julius & Nesbitt, 1996). This is supported

by epidemiological studies that the upregulated sympathetic tone is found in hypertensive patients since their childhood (Malpas, 2010). Also, recent data from the Birren lab show that cardiac hyperinnervation exists in spontaneously hypertensive rats (SHR) compared with their normotensive control Wistar-Kyoto (WKY) rats (Huang, 2018). Multiple mechanisms are involved in the development of hypertension related to overactivity of the SNS, including high levels of intrinsic neuronal modulators such as neuronal nitric oxide synthase (nNOS), as well as increased sympathetic neural co-transmitter induced elevated adrenergic drive caused by neuropeptide-Y, ATP and galanin (Wang and Golledge, 2013; Calvillo et al., 2019).

3) Chemical sympathectomy and current modes of action

Chemical sympathectomy, unlike traditional surgical sympathectomy, where a surgeon cuts or clamps sympathetic nerve chain, involves the usage of chemical agents to remove or diminish effects from sympathetic stimuli (Rascher et al., 1983). Chemical sympathectomy is selected as a denervating approach in this study, in order to test the hypothesis that early reduction or removal of elevated sympathetic drive in SHR can prevent development of a later hypertensive state. 6-hydroxydopamine (6-OHDA), a widely used neurotoxin, serves as a selective sympathetic denervation tool in this study. 6-OHDA can be selectively taken by catecholamine nerve terminals because as a dopamine congener it enters cells via monoamine transporters (Joers et al., 2014). There are two main mechanisms explaining the neurotoxicity at the molecular level (Sachs & Jonsson, 1975): firstly, oxidation products of 6-OHDA tend to bind nucleophilic groups of macromolecules such as SH_2, NH_2, and phenolic OH, which damages integrity of neurons. Secondly, free radicals produced along with the oxidation of 6-OHDA are selectively taken up and

concentrated at the presynaptic membrane of catecholamine neurons and promote oxidative stress resulting in cytotoxic effects.

4) Model organisms

SHR is the most studied animal model of essential hypertension, commonly used to study cardiovascular disease. The model is derived from the outbred WKY rats with marked elevation of blood pressure (Pinto et al., 1998). The SHR model recapitulates many pathophysiological characteristics for essential hypertension in human including onset timing, sex differences and major internal organs involvement such as heart and kidney. The normotensive WKY rats serve as control, whereas SHR model was derived by selective breeding of WKY rats that spontaneously developed elevated blood pressure (Panzenhagen et al., 2019). Therefore, WKY rats are studied in comparison with SHR to investigate perturbations in SHR strain. Since at 5-6 weeks of age, SHR start to develop hypertensive characteristics, the intended time points of study are 4 weeks (prehypertensive state), 8 weeks (early hypertensive states), and 12 weeks (hypertensive state). These cover life period of the animal that will provide ground for establishing a developmental model for hypertension. Importantly, it includes life period of prehypertensive state which is relevant since our finding indicate that hyperinnervation occurs before hypertension onset. There is also evidence suggesting that heightened sympathetic activity proceeds the hypertension onset (Thorp & Schlaich, 2015).

5) Reasons for chosen approaches

To test the hypothesis that early reduction or removal of elevated sympathetic drive can prevent the development of hypertensive state, both physiological and morphological read-outs were carried out and evaluated. Non-invasive tail-cuff methods for measuring of blood pressure (BP) were used at ages of 8 weeks and 12 weeks. Although invasive intraarterial cannulation has been viewed as gold standard for BP recording, emerging evidence shows strong correlation between non-invasive BP measured by the tail-cuff method and the intraarterial method (Gangwar et al., 2014). To observe effects of chemical sympathectomy, superior cervical ganglia (SCG), which is part of the SNS, and hearts from the sympathectomized and sham rats were analyzed histologically at time points of 4, 8 and 12 weeks of age.

In this study, I evaluated the role of sympathetic drive in the development of hypertension by reducing or removing the sympathetic drive via neonatal sympathectomy. I found that neonatal sympathectomy induced by 6-hydroxydopamine (6-OHDA) diminished cardiac sympathetic innervation and reduced blood pressure of 12-week-old SHR. This indicates existing bonds between cardiac sympathetic hyperinnervation and promoted development of hypertension. Taken together, this study provides evidence that early neonatal intervention directed towards modulation of sympathetic cardiac innervation can influence a course of essential hypertension development.

Materials and Methods

Ethical approval

Spontaneously hypertensive rats (SHR), Wistar Kyoto (WKY) rats, and Sprague-Dawley (SD) rats were purchased from Charles River Laboratories and raised and bred in Brandeis animal facility. The sympathectomy of SHR and WKY rats, and all animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of Brandeis University approved protocol (protocol #19008).

Chemical sympathectomy

Chemical sympathectomy was performed on both SHR and WKY rats by subcutaneously (s.c.) administering 100 mg/kg of 6-hydroxydopamine (6-OHDA) (Sigma-Aldrich, Burlington, MA, USA) per day for a period of 4 consecutive days commencing from postnatal day 1 (P1). To prevent 6-OHDA from oxidation, ascorbic acid was added (Wang et al., 2017). Thus, administered 6-OHDA was dissolved in saline solution with ascorbic acid (2 mg/mL). Animals (control group) injected with the ascorbic acid in saline served as sham. Animals injected with 6-OHDA (i.e. sympathectomized) were tattooed on rear paws with permanent green ink for identification.

Non-invasive measurement of blood pressure

Blood pressure (BP) was measured with a non-invasive tail-cuff blood pressure measuring system (CODA 8, Kent Scientific, Torrington, CT, USA). At 8 or 12 weeks of age, animals were acclimatized to a CODA 8 system, with restricted animal movement and infrared heated platform

(Kent Scientific, Torrington, CT, USA). BP of each animal was measured on at least two separate days. For each day, rats were firstly acclimated to the system for 20 mins followed by data collection. In order to optimize measurement, each data set collection included 5 defaulted unaccepted cycles and 15 experimental cycles. At least 5 visually verified, reliable/undisturbed measurements of these 15 cycles were used for BP calculation.

Tissue collection

Heart and superior cervical ganglia (SCG) were isolated from SHR and WKY rats for the three age groups (4 weeks, 8 weeks and 12 weeks) after the CO_2 euthanasia. Hearts were perfused with phosphate buffered saline (PBS) in order to minimize contribution of blood contamination to autofluorescence of imaged samples. Once perfusion was complete, heart and SCG were collected from animals. Body weight and heart weight were assayed at the point of euthanasia.

Tissue processing

Collected hearts and SCG were fixated in 4% paraformaldehyde (PFA) at 4˚C for at least 12 hours. After fixation, tissue was washed 3 times with PBS, each wash lasted 15 minutes. After the third wash, tissue was transferred into 30% sucrose for cryopreservation. Once completed, the tissue was then removed from sucrose and placed in cryomolds filled with Optimal Cutting Temperature (OCT) compound. Additionally, SCG were stained with TrueBlack™ (Gold Biotechnology®, St. Louis, MO, USA) before transferring into molds, to delineate borders of SCG and differentiate the SCG from surrounding compound. The mold with SCG was frozen on dry ice for 5-10 mins, until the block turned opaque, which would then be stored at -80˚C until further use (cryosectioning).

Cryosectioning

Tissue was then cut into 10 µm, longitudinal sections by the cryostat (Leica CM3050, Buffalo Grove, IL, USA) and 2-4 sections were mounted on a clear slide. The slides were labeled and stored at -20˚C in a slide box.

Immunohistochemistry (IHC) for hearts

Slides were rehydrated in PBS for 30 minutes and the excess of PBS was wiped out. Borders encircling tissue were drawn on the slides using ImmEdge Hydrophobic Barrier Pen (Vector Labs, Inc., Burlingame, CA, USA). In order to reduce background immunofluorescence, slides were treated with sodium borohydride solution (10 mg/mL), which was repeated thrice 10 minutes each. Slides were then washed using PBS thrice 10 minutes each and then were blocked by blocking buffer (3% Bovine serum albumin/ 0.3% Triton X-100 in PBS) shaking for an hour. The encircled parts of tissue sections were then incubated with primary antibodies (Chicken anti tyrosine hydroxylase antibody, ab76442, 1:1000 dilution) (Abcam, Cambridge, MA, USA) overnight at room temperature. After three-time 10 minutes wash using PBS, sections were incubated with secondary antibodies (Donkey anti chicken rhodamine, 1:500) (Invitrogen, Carlsbad, CA, USA) for 1.5 hours at room temperature. Slides were then washed thrice using PBS and dipped into double-distilled water once for 3 seconds before sealed with clear nail polish in 20 µL glycerol in 50% PBS.

Immunohistochemistry (IHC) for SCG

The slides were rehydrated in PBS for 30 minutes and the borders were drawn by ImmEdge Hydrophobic Barrier Pen (Vector Labs, Inc., Burlingame, CA, USA). Subsequently, the slides were chilled on ice and treated with sodium borohydride solution (10 mg/mL) to reduce autofluorescence, 3 times for 10 minutes each. After 3 times wash with PBS, the slides were incubated with blocking buffer (3% Bovine serum albumin/ 0.3% Triton X-100 in PBS), shaking for 1 hour. SCG sections were then incubated with primary antibodies at room temperature overnight. The detailed are as follows: chicken anti-Microtubule Associated Protein 2 (MAP2) polyclonal antibody (Sigma- Aldrich, EMD Millipore, Darmstadt, Germany, AB553, 1:1500); rabbit anti-S100 calcium-binding protein B subunit β polyclonal antibody (Agilent Dako, Santa Clara, CA, USA, Z0311, 1:400). The excess nonspecifically bound antibodies were washed away using PBS 3 times for 10 minutes each. Then, the slides were incubated with secondary antibodies: donkey anti chicken rhodamine and donkey anti rabbit Alexa 488 (Invitrogen Life technologies, Carlsbad, CA, USA) in the dark at room temperature for 1.5 hours. Still under dark conditions, the slides were then rinsed with PBS to remove any nonspecifically bound secondary antibodies and then incubated with 1 mg/mL 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Invitrogen Life technologies, Carlsbad, CA, USA, 1:20) for 20 minutes. Subsequently, the slides were rinsed 2 times, 10 minutes wash each, with PBS to remove the nonspecifically bound DAPI. Lastly, the slides were sealed with clear nail polish for confocal microscopy.

Confocal microscopy

Both heart and SCG sections were imaged using Zen software by a laser scanning confocal microscope (Zeiss 880, White Plains, NY, USA) at 20 x or 40 x magnification depending on sizes of the sections.

Cell density and morphology quantification

Three SCG sections per animal and 2-4 images per section were taken using the 561 nm, 488 nm and 405 nm lasers to excite the three fluorochromes: rhodamine, Alexa 488, and DAPI, respectively. Neurons in SCG sections were identified by MAP2 staining, glial cells by S100β staining, and nuclei by DAPI staining. The number of neurons was counted using the Cell Counter plug-in of the Fiji (SciJava Consortium) software. Using this method, neurons and glia were analyzed from three SCG and about 27 sections per condition and neuron size and cell density was determined from the maximal projection of the Z-stacks. Cells stained for both S100β and DAPI were identified as glial cells and the number and density of glial cells was calculated. Counting glial number is difficult due to the ensheathment properties of the cells. Briefly, we developed (and have made available on GitHub, search for Enes2020; https://github.com/birrenlab/Enes2020), a MATLAB™ script to determine the number of glial nuclei in each image by calculating the total DAPI-positive-S100-positive double-labeled area and dividing that number by the median area of DAPI and S100-co-stained nuclear structures. This number represents the total number of glial nuclei associated with S100 staining. This was then divided by the total area of the field being counted to obtain a measure of glial density (defined as glial nuclei per mm^2) in each section. The

neuron soma size was measured by manually outlining MAP-2 stained neurons within a rectangular area of identical size and position in each image using Fiji software in sections stained for MAP2, S100β and DAPI.

Statistics

All data are presented as the mean of at least three independent experiments ($n \geq 3$) with the standard error of the mean indicated by error bars. To determine statistical significance, Student's t-test was used for two groups and ANOVA along with appropriate post-hoc tests were selected for over two groups. Data were compiled and analyzed in Excel (Microsoft, Seattle, WA, USA) or Prism 8 (GraphPad software, San Diego, CA, USA).

Results I- Left ventricular hyperinnervation and hypertension

Multiple 6-OHDA injections do not result in heart weight differences in prehypertensive SHR and WKY rats.

To investigate effects of chemical sympathectomy on postnatal heart development, I conducted four 6-OHDA and sham s.c. injections administered each consecutive day, beginning at postnatal

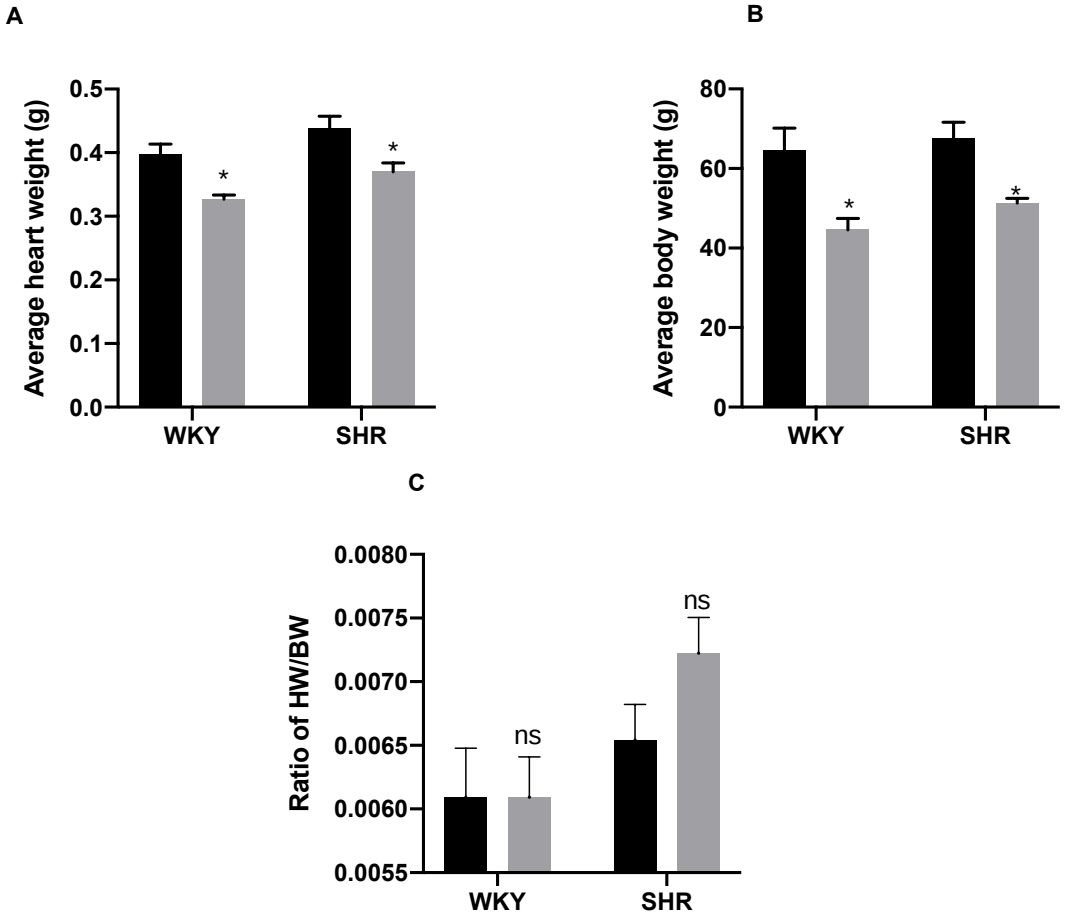

Figure 1 Effects of reinforced sympathectomy on heart and body weight in prehypertensive WKY and SHR rats. Comparisons between sham treated (black) and 6-OHDA lesioned (grey) 4-week-old WKY and SHR in average heart weight (HW), average body weight (BW) and ratio of HW/BW. A. Heart weights were significantly reduced by chemical sympathectomy in both strains as compared to sham control treatments. B. 6-OHDA induced sympathectomy resulted in a significant decrease in average body weight. C. Ratio of HW/BW in sham treated and sympathectomized WKY and SHR. (WKY sham n=5, WKY lesion n=3, SHR sham n= 6, SHR lesion n=4; One-way ANOVA with Tukey 's multiple comparisons test was used. *$p < 0.05$ *vs.* strain matched control)

day 1 (P1) of WKY rats and SHR. Heart weight and body weight were determined in 4 weeks old rats. The neonatal sympathectomy (reinforced four-times, induced by daily injections of 6-OHDA administered to P1 pups through their P4), decreased average heart weight in both strains. Likewise, multiple 6-OHDA injections reduced average body weight for both SHR and WKY rats. This is consistent with published data, and suggestive of a loss of body weight along the course of 6-OHDA treatment (Gálosi et al., 2015). However, once heart weight is normalized to body weight, differences in the calculated ratio values between lesioned and sham injected SHR or WKY rats are not significant. In addition, there is no significant difference between SHR and WKY rats at the age of 4 weeks old in heart weight, body weight or ratio of heart weight to body weight.

In summary, multiple chemical sympathectomy induced by subcutaneous administration of 100 mg/kg of 6-OHDA from P1 to P4 does not result in a significant heart weight change relative to body weight between SHR and WKY rats at 4-weeks of age.

Does neonatal chemical sympathectomy reduce heart sympathetic innervation in the adult?

I next asked whether 6-OHDA treated SHRs have decreased sympathetic innervation of the heart

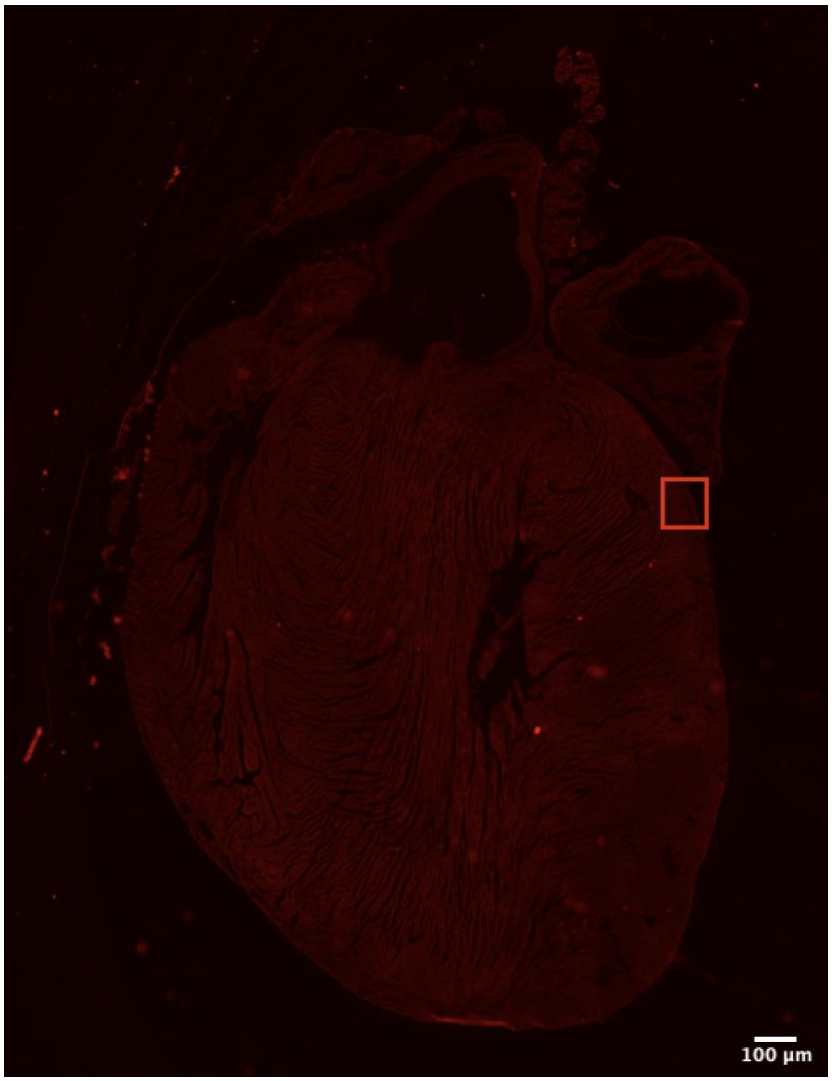

Figure 2 A representative stitching image of longitudinal heart section. Boxed area indicates part within the left ventricle, where the innervating density analysis was determined. Sympathetic innervation was evaluated by immunohistochemical tyrosine hydroxylase (TH)staining of sympathetic neuronal fibers. Scale bar = 100 µm.

compared with their sham-injected controls. To evaluate this, I focused my attention on innervation of the heart muscles surrounding the left ventricle, since the published data suggested that sympathetic innervations of the left ventricular wall play a critical role in the pathogenesis of cardiovascular diseases (Vaseghi et al., 2012). I therefore analyzed the sympathetic fiber density

of at least 3 different sections per heart, namely the anterior epicardium of the cardiac left ventricle, in 3 hearts per condition were examined (see red box area in Figure 2).

I examined the density of sympathetic innervation at the age of 4 weeks, 8 weeks and 12 weeks. To determine nerve fiber density, heart sections were stained for tyrosine hydroxylase (TH) and analyzed with FIJI to calculate out the innervating density (Figure 3 & 4).

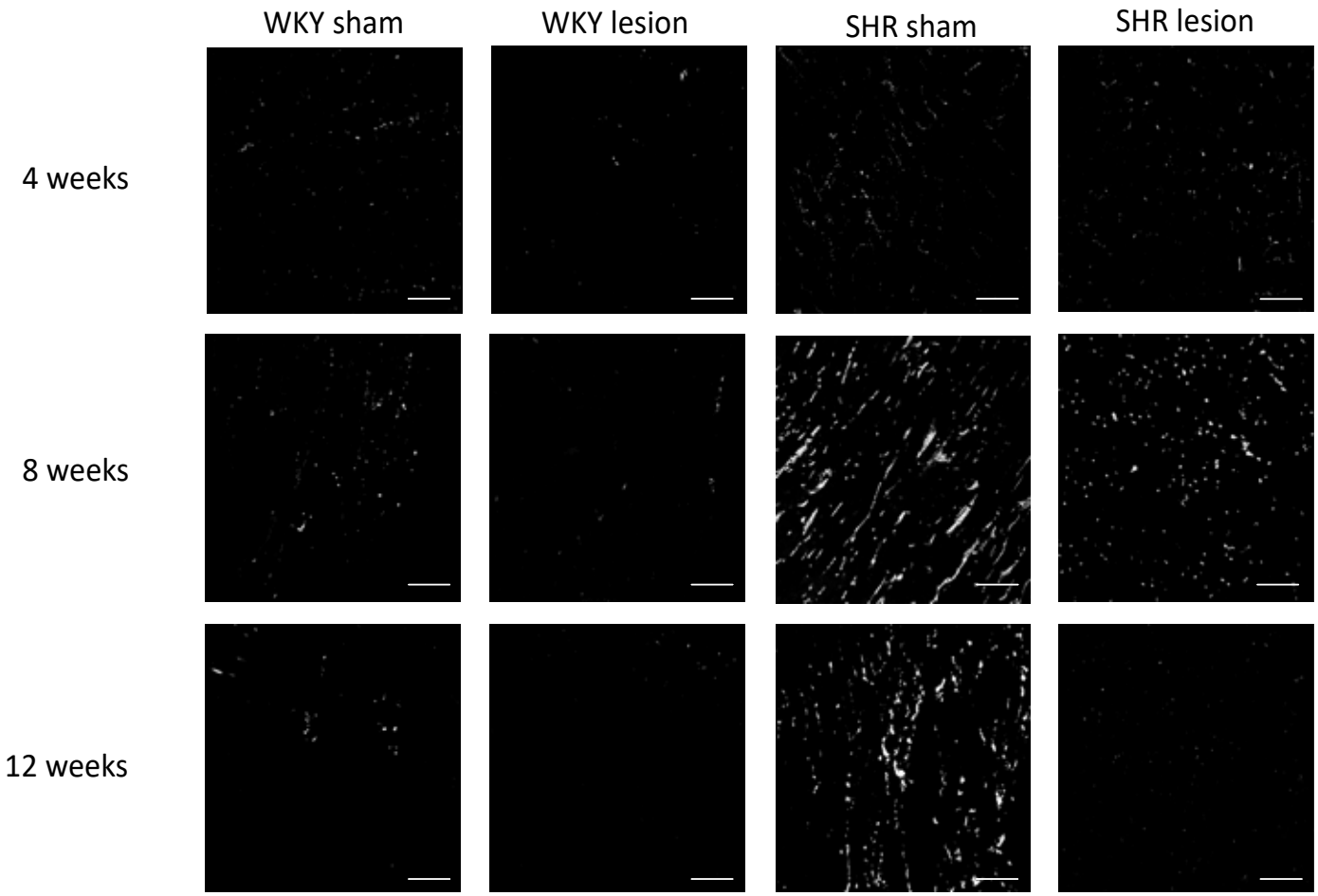

Figure 3 Representative IHC images demonstrating cardiac sympathetic innervation. Imaged sections were taken at anterior epicardium of cardiac left ventricles from 4-week-old, 8-week-old and 12-week-old SHR or WKY rats with 6-OHDA treatment (lesion) or vehicle control (sham). Sympathetic fibers were determined by TH staining. Scale bar = 10 μm.

Comparisons of 6-OHDA treatments (lesion) with vehicle control (sham) for WKY and SHR strains showed decreases in left ventricular innervation at all age groups examined (row 1= 4 weeks, row 2 = 8 weeks, and row 3 = 12 weeks). The white discontinuous traces of TH staining are visibly more reduced in 6-OHDA lesions than in shams, for both WKY and SHR.

The highest density of innervation was found at 8 weeks in SHR sham group. The WKY lesion and SHR lesion group also peaked at 8 weeks. Figure 4 provides detailed averaged quantifications. Innervation went up first at 8 weeks, and then dropped down at 12 weeks in these groups (Figure 5), with the exception of the WKY sham group. It is indeed not surprising to see changes in innervation of the tissue during development. Kreipke and Birren found marked reduction of innervating density from P2, P7 to 8-week-old SD rats at the heart left ventricles (Kreipke & Birren, 2015). Additionally, Chartier et al. detected a 75% reduction of sympathetic nerve fiber density in the adult and aging cortical bone of mice femur (Chartier et al., 2018).

Our data are consistent with earlier observations of Huang (Huang, 2018), in which he compared sympathetic innervation density of heart in SHR and WKY rats at P2, P7, P14 and 4 weeks, and observed increased innervation density in hearts of SHR. Our data here showed similar trends, that is, SHRs demonstrates a higher cardiac sympathectomy innervating density than that of their normotensive controls, WKY rats, at the time points of 4 weeks, 8 weeks and 12 weeks (Figure 4).

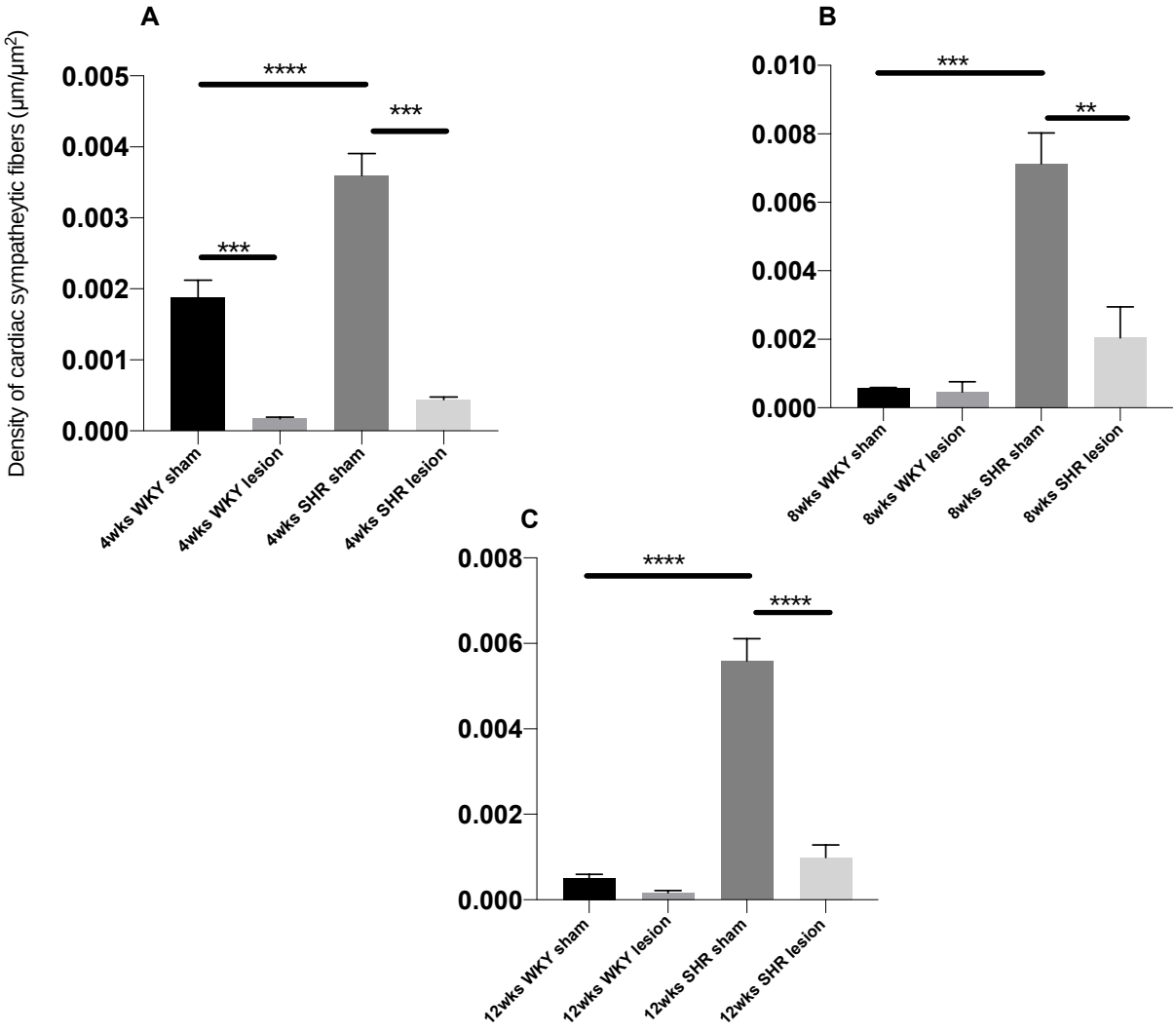

Figure 4 Innervation of heart left ventricle by sympathetic fibers. Average densities of sympathetic fibers at different age groups: A (4 weeks), B (8 weeks), and C (12 weeks). TH staining was calculated from three different hearts (n=3) and data were analyzed by one-way ANOVA followed by Sidak's multiple comparisons test. Data represents mean ± SEM (**p＜0.01, ***p＜0.001, ****p＜0.0001）

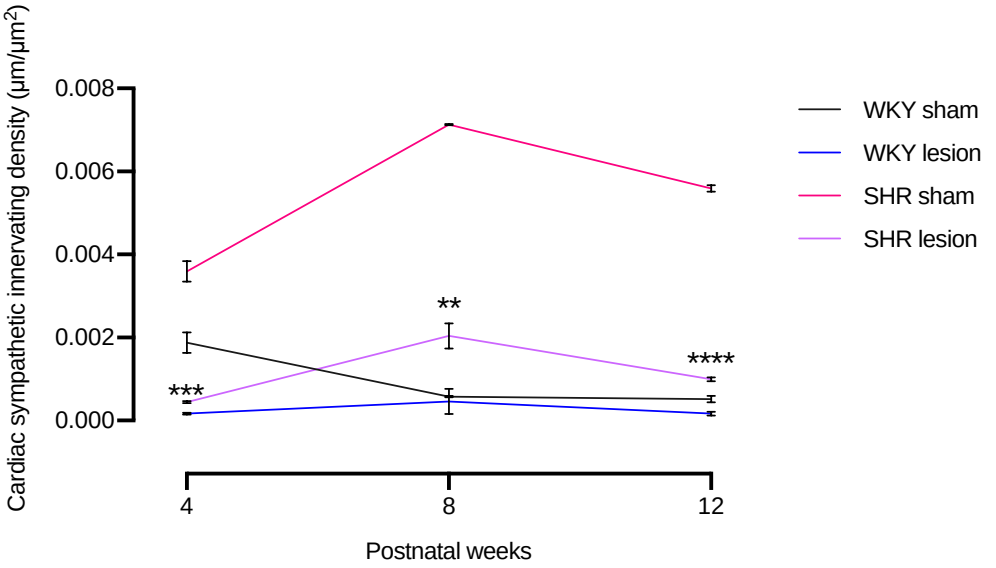

Figure 1 Developmental changes of cardiac sympathetic innervating density. Quantification of average density of left ventricular innervation from analyzing heart sections of WKY sham (black), WKY lesion (blue), SHR sham (magenta) and SHR lesion (lavender). Data were represented as Mean ± SEM. * denotes significant difference of SHR lesion *vs.* SHR sham. (**p＜0.01, ***p＜0.001, ****p＜0.0001)

Interestingly, following the 6-OHDA sympathectomy, 4-week-old SHRs even showed a lower fiber density than that of their WKY control, which indicates that effective 6-OHDA induced sympathectomy could lower sympathectomy fiber density below the level of their normotensive controls. However, this is not the case for either 8 weeks or 12 weeks old WKY lesion groups compared with their sham-treated controls.

In summary, our data indicate that 6-OHDA induced sympathectomy in neonatal SHR animals was very effective in decreasing innervating fiber density of the left ventricle in 4-week-old SHRs. Indeed, sympathetic fiber density was decreased in 4-week-old animal group even below the level of their normotensive controls. Also, physiologically relevant might be the fact, that decreased

innervation density of the left ventricle persists in the 8- and 12- week old SHR 6-OHDA treated groups.

Can neonatal sympathectomy prevent development of increased blood pressure in SHRs?

It is well-established that elevated sympathetic drive leads to essential hypertension (Voora & Hinderliter, 2018). However, the role of cardiac hyperinnervation in the development of essential hypertension still remains to be explored. In order to examine effects of chemical sympathectomy (reinforced four-times, induced by daily injections of 6-OHDA administered to P1 pups through their P4) on the development of hypertension, blood pressure (BP) in SHR and WKY rats was measured using a non-invasive tail cuff method.

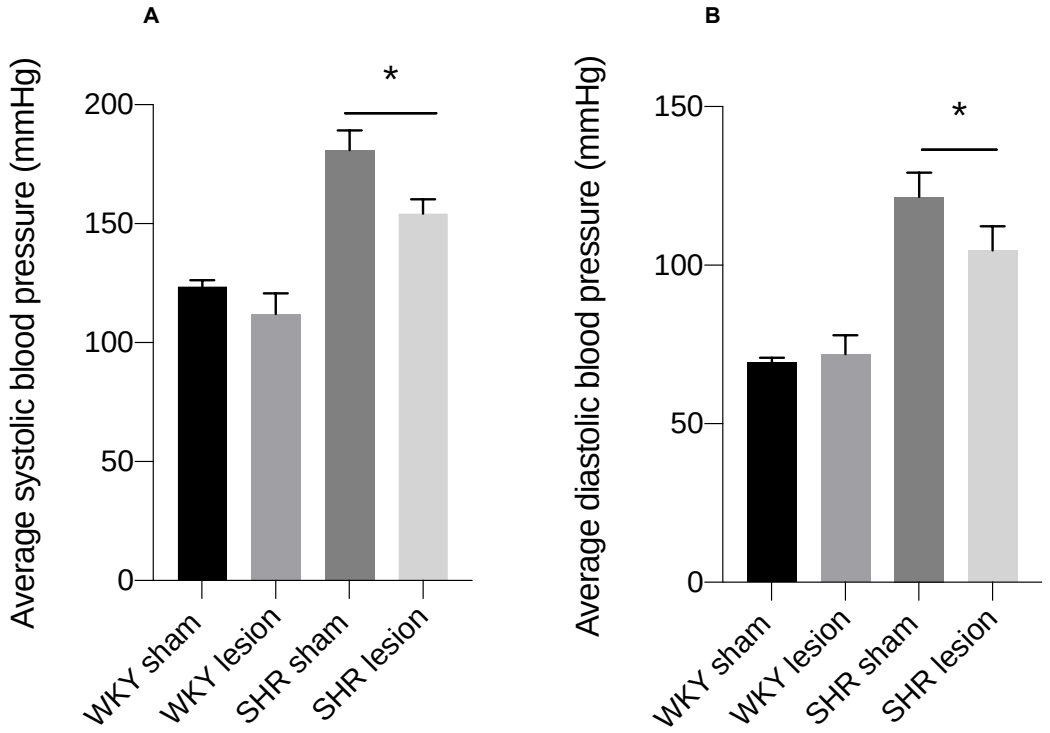

Figure 6 Neonatal chemical sympathectomy reduces the blood pressure of 12-week-old SHR. A. Average systolic blood pressure measured from WKY sham, WKY lesion, SHR sham and SHR lesion groups. B. Average diastolic blood pressure derived from WKY sham, WKY lesion, SHR sham and SHR lesion groups. Data presented are Mean ± SEM. Unpaired student's t test was performed to test for significance, * denotes level of $p < 0.05$. Sample size n of WKY sham, WKY lesion, SHR sham and SHR lesion are 3, 3, 5, and 4, respectively.

Both systolic and diastolic blood pressure were found to be reduced (Figure 6) by neonatal multiple chemical sympathectomy in 12 weeks old SHRs. The SHR lesion group (6-OHDA treated) demonstrated significantly lower average systolic blood pressure compared with their sham-treated controls (Figure 6A, (154.1 ± 6.0 mmHg) *vs.* (180.9 ± 8.3mmHg), $p < 0.05$). Likewise, average diastolic blood pressure of 12-week-old SHR lesion group was diminished (Figure 6B, 104.6 ± 7.7 mmHg *vs.* 121.6 ± 7.6 mmHg, $p < 0.05$). These changes indicate that 6-OHDA induced

chemical sympathectomy effectively lowered systolic and diastolic blood pressure in 12-week-old SHRs.

For the 12-week-old WKY rat group, 6-OHDA treatments did not result in significant decrease in either systolic or diastolic blood pressure (see first two columns in Figure 6A and 6B).

Does 6-OHDA induced sympathectomy modify the function of neurons and/or satellite glia within SCG?

As some of the neuronal sympathetic fibers innervating heart are processes of neurons from nearby peripheral sympathetic ganglia, including SCG, I was curious to evaluate the effect of sympathectomy on cells within those ganglia. In particular, besides expected effects on neurons within ganglia, I investigated whether the 6-OHDA-induced sympathectomy might induce cytotoxic effect on sympathetic neurons and satellite glia, which could lead to the initiation of pathological response of SCG of SHR.

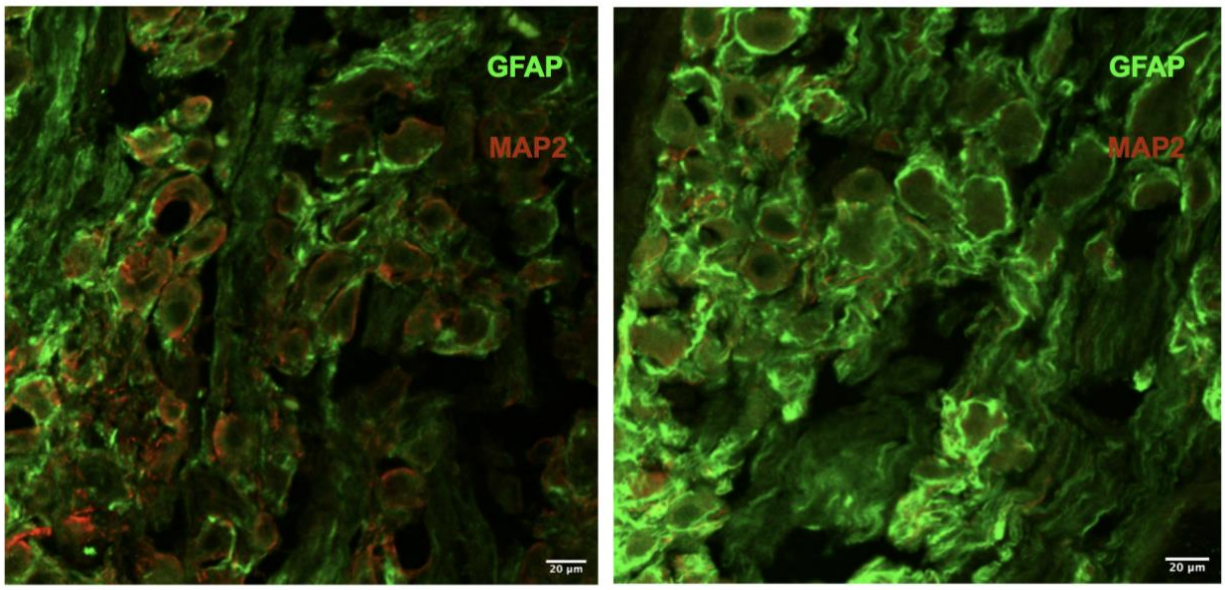

Figure 7 Immunohistochemistry of SCG of sham and 6-OHDA treated SHR. Representative images of SCG sections from 8-week-old sham (left panel) and 6-OHDA treated lesioned (right panel) SHRs. Sections were stained against a neuron specific marker MAP2 and a glia specific marker GFAP. Scale bar = 20 µm.

As a preliminary experiment, I delved in 8-week-old SHR rats with or without chemical sympathectomy. Since Glial Fibrillary Acidic Protein (GFAP) is a marker for activated glia, I asked if chemical sympathectomy led to activated glia induced initiation of SCG pathology. The glial activation in the central nervous system has been seen in various pathological states (Garden & Campbell, 2016). However, the role of the peripheral satellite glia is less understood.

Confocal images of SCG sections from 8-week-old SHR treated with 6-OHDA (Figure 7, right panel) and vehicle treated (sham) (Figure 7, left panel) stained by glia specific marker Glial Fibrillary Acidic Protein (GFAP-green) and neuron specific marker Microtubule-associated Protein 2 (MAP 2-red). Visibly, more green elements enwrapping red round-shaped neurons are found in the right panel compared with the control in the left panel, indicating more glial cells were activated. The preliminary data suggest that multiple 6-OHDA injections might induce the initiation of pathological responses in the SCG of SHR.

Results II- Developmental changes of neurons and glia in SCG

Dynamic changes in the ganglionic structure of sympathetic neurons and satellite glia during the postnatal period

With the focus on interaction between sympathetic neurons and glia in the SCG, I next asked whether the close communication between these cells could promote dynamic morphological changes in early developmental stages. I stained P2 and P21 SCG

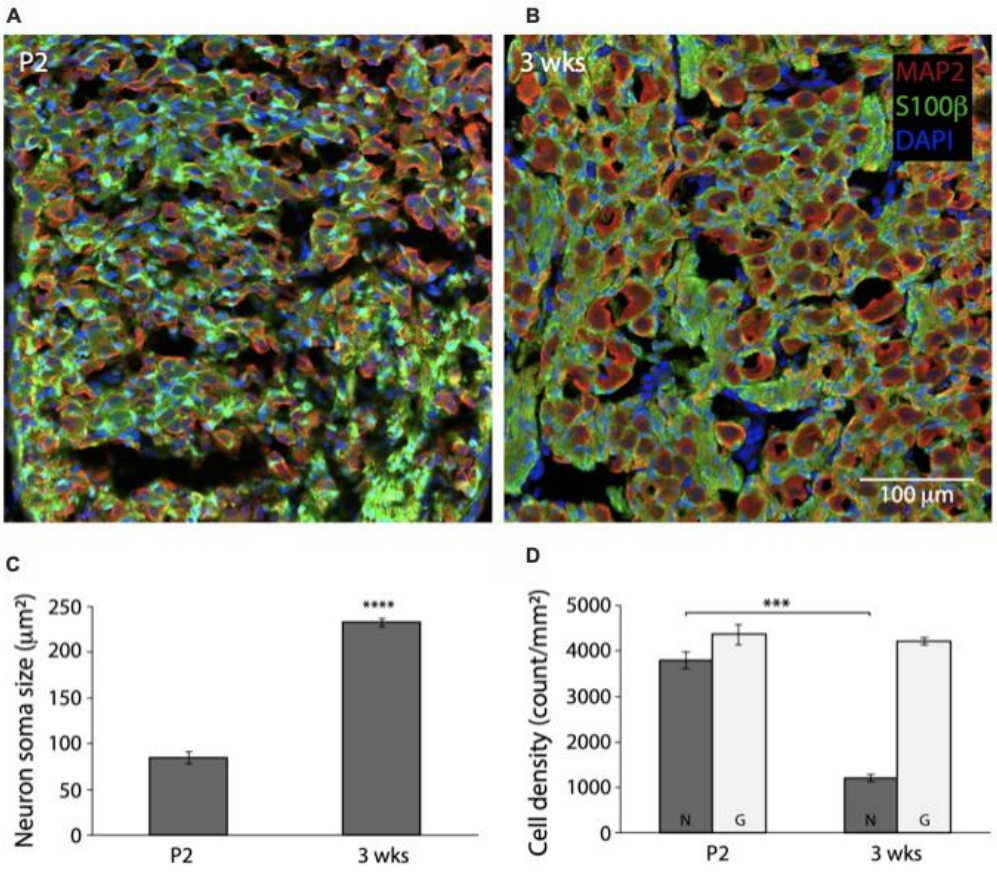

Figure 8 Developmental changes of neurons and satellite glial cells in the SCG A-B, representative IHC images of P2 (A) and P21(3 weeks) (B) SCGs from SD rats. Sections were stained for the neuron-specific marker MAP2 (red), satellite glial cells for the glial cell marker S100β (green) and cell nuclei using DAPI (blue). Scale bar = 100 µm. C-D, Quantification of (C) neuron soma size, measured as average cell area in the section and (d) neuronal and glial cell densities from sections of P2 (n=3; mean ± SEM) and 3 wks (n=3; mean ± SEM). ***p<0.001, **p<0.01, *p<0.05 determined by ANOVA followed by Tukey's HSD comparison test.

sections of Sprague Dawley (SD) rats with MAP 2 to identify neuronal soma and S100β to identify glia (Fig 8A-B). I observed a close juxtaposition between neurons and glia at both time points, even as the morphology of the ganglia changed.

Between P2 and P21, sympathetic neurons increased in size (Fig 8C) as the neurons make target contacts and are exposed to target derived signals that promote cellular hypertrophy. As neurons increase in size the number of neurons per unit area decreases, while the density of satellite glial cells remains constant over the first three postnatal weeks (Fig 8D). Note: This data is included in the following publication:

Enes J, Haburčák M, Sona S, Gerard N, Mitchell AC, **Fu W**, Birren SJ.: Satellite glial cells modulate cholinergic transmission between sympathetic neurons. PLoS One. 2020 Feb 4;15(2):e0218643. doi: 10.1371/journal.pone.0218643.

Discussion and Future directions

The aim of this study was to investigate effects of neonatal sympathectomy on the development of essential hypertension, which is intimately related to cardiac sympathetic hyperinnervation, heart physiological or pathological characteristics such as blood pressure and cardiac hypertrophy. Fully blown hypertension is observed in SHR by the 12th week of age, while a prehypertensive status already observed in 5th-6th week. My results showed that by disrupting hyperinnervation at an early age neonatally sympathectomized SHR hypertension was disrupted and brought close to the normal level observed in age-matched WKY rats. In addition, both systolic and diastolic blood pressure of 12-week-old SHR was moderately reduced following the 6-OHDA induced denervation.

6-OHDA induced sympathectomy did not cause significant heart weight changes between SHR and WKY 4-week-old rats. Heart weight to body weight ratio is widely used as a measure of hypertrophy (Aronow, 2017). Since we hypothesized that 6-OHDA would reverse the development of essential hypertension in SHRs, it was reasonable to predict that following chemical sympathectomy, the left ventricular hypertrophy observed in SHRs could be reversed. Specifically, this means a reduced HW/BW ratio after the 6-OHDA treatment. However, 6-OHDA did not cause a significant decrease in HW/BW ratio. One possibility is that at the early stage, cardiac hypertrophy in SHR was significantly diminished by 6-OHDA sympathectomy but recovered afterwards by the 4th week of age. This is supported by the published data that neonatal sympathectomy effectively decreased heart size of P2 and P7 rats (Kreipke & Birren, 2015), despite differences in days of injections, drug administrating route and animal models. To

adequately conclude the effect of sympathectomy on heart hypertrophy index, more time points, including animals younger than 4 weeks of animals age should to be chosen for HW/BW ratio assays. Another possibility is that the 4-time 6-OHDA injections used for sympathectomies in these experiments did not exert a strong, observable effect on heart hypertrophy (Finch et al., 1973).

My examinations of the left ventricular innervation showed no significant differences after the treatment for 8-week-old and 12-week-old WKY rats. Nevertheless, there are relatively sparse fibers detected, particularly when reduced by performed chemical sympathectomy. The tracing tools developed for FIJI might be not adequate for catching subtle differences in fiber length and might also be affected by observer biased error. If so, more effective tracing tools might be used in the future.

In the future, experiments that were first disrupted by the heat crisis in August 2019 and now by the laboratory shutdown in response to COVID-19 need to be completed. These include blood pressure measurement in 8- and 12-week-old animals to compare developmental changes in blood pressure with or without chemical sympathectomy. Additionally, since sexual dimorphism exists in the development of hypertension, a lag phase may exist in the development of hypertension in females (Sandberg & Ji, 2012). Thus, experimental plans should include the need for dissecting sex differences in developmental changes in hypertension. There are also experiments proposed to explore factors contributing to the detected sympathetic hyperinnervation. This might be happening within the ganglia, by local interaction between cells within ganglia (e.g. neuron-satellite glia modulation) (Enes et al., 2019). Since the SCG are sources of cardiac sympathetic fibers, the interaction is not limited to transmission of signaling molecules between neurons and

satellite glial cells in the SCG only, but also directly at the site of interaction within target organ. It is indeed acknowledged that some of the growth factors are produced by cardiac myocytes and might even be retrogradely transported to SCG (Lockhart et al., 1997). These factors may play a critical role in the development of cardiac sympathetic innervation and can modulate cholinergic transmission of sympathetic neurons. It was reported that P75, as a low affinity NGF receptor, is critical to the cholinergic transmission of sympathetic neurons (Luther and Birren, 2009). Thus, IHC staining for P75 in SCG sections might provide valuable information and reveal a new role for these receptors in the state of developing hypertension.

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